Genetic Analysis of the Invariant Residue G791 in *Escherichia coli* 16S rRNA Implicates RelA in Ribosome Function

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Received 1 July 2008/Accepted 6 January 2009

Previous studies identified G791 in *Escherichia coli* 16S rRNA as an invariant residue for ribosome function. In order to establish the functional role of this residue in protein synthesis, we searched for multicopy suppressors of the mutant ribosomes that bear a G-to-U substitution at position 791. We identified relA, a gene whose product has been known to interact with ribosomes and trigger a stringent response. Overexpression of RelA resulted in the synthesis of approximately 1.5 times more chloramphenicol acetyltransferase (CAT) protein than could be synthesized by the mutant ribosomes in the absence of RelA overexpression. The ratio of mutant rRNA to the total ribosome pool was not changed, and the steady-state level of CAT mRNA was decreased by RelA overexpression. These data confirmed that the phenotype of RelA as a multicopy suppressor of the mutant ribosome did not result from the enhanced synthesis of mutant rRNA or CAT mRNA from the plasmid. To test whether the phenotype of RelA was related to the stringent response induced by the increased cellular level of (p)ppGpp, we screened for mutant RelA proteins whose overexpression enhances CAT protein synthesis by the mutant ribosomes as effectively as wild-type RelA overexpression and then screened for those whose overexpression does not produce sufficiently high levels of (p)ppGpp to trigger the stringent response under the condition of amino acid starvation. Overexpression of the isolated mutant RelA proteins resulted in the accumulation of (p)ppGpp in cells, which was amounted to approximately 18.2 to 38.9% of the level of (p)ppGpp found in cells that overexpress the wild-type RelA. These findings suggest that the function of RelA as a multicopy suppressor of the mutant ribosome does not result from its (p)ppGpp synthetic activity. We conclude that RelA has a previously unrecognized role in ribosome function.

rRNA accounts for more than 60% of ribosomal mass and plays an essential role in the catalytic process in translation. Highly conserved sequences exist in rRNAs, and the 790 loop (positions 787 to 795) in small-subunit (SSU) rRNA is one notable example (2). This loop has been shown to be heavily involved in subunit association (20, 35). Residues in the 790 loop are protected from chemical probes through the binding of initiation factor 3 (G791 and U793) (25, 26), 50S subunits (A790 and G791) (23), and P-site-bound tRNA<sup>Met</sup> (A794 and C795) (8, 23), as well as by the antibiotics kasugamycin (A794), pactamycin (C795), and edeine (A794 and C795) (8, 22, 24). The crystal structure of the 30S subunit places the 790 loop in the front half of the platform, where the 790 loop forms bridges of electron density that extend toward the 50S subunit in the 70S ribosome crystal structure (4, 5, 38, 43).

By utilizing a novel genetic approach termed “instant evolution,” which involves random mutagenesis of all of the nucleotides in the 790 loop and in vivo selection of functional alternative sequences, Lee et al. (20) identified invariant nucleotides that may be involved in ribosomal functions by interacting with ligands. One such residue, G791, is very well conserved in SSU rRNAs (2). To identify the functional role of this residue, we adopted a genetic approach that overexpressed mutant ribosomes bearing a base substitution at position 791 through the specialized ribosome system (pRNA122) (20, 21) as well as *Escherichia coli* proteins from genomic library clones. The genetic screen identified relA as a multicopy suppressor that partially enhanced the protein synthesis ability of the mutant ribosome. The relA gene product synthesizes GTP 3'-diphosphate (pppGpp) and guanosine 3',5' biphosphate (pppGpp), which are collectively referred to as (p)ppGpp (13, 34). The synthesis reaction occurs when the binding of uncharged tRNA to the aminocyl-tRNA site (A site) is recognized by RelA, which interacts with ribosomes (12), and this consequently triggers an adaptation response in many bacteria. This response is termed the stringent response (18). The stringent response involves the transcriptional repression of genes associated with the translational apparatus (18) and the up-
regulation of genes that encode metabolic enzymes, especially those involved in amino acid synthesis (3).

We examined the effects of RelA overproduction on protein synthesis by both the wild-type ribosome and the specialized ribosome that bears an original residue or a base substitution at position 791. By utilizing the specialized ribosome system and an expression system for mutant RelA proteins that contain random amino acid substitutions, we further isolated mutant RelA proteins that do not synthesize sufficiently high levels of (p)ppGpp to permit normal growth of E. coli cells in the presence of 15 mM 3-amino-1,2,4-triazole (AT), a histidine analog which induces histidine starvation but which still complements the mutant ribosome bearing a base substitution at position 791. We concluded from our results that the high levels of (p)ppGpp produced by RelA were unlikely to enhance the protein synthesis capabilities of the mutant ribosome. Based on these findings, we suggest an involvement of RelA in ribosome function.

MATERIALS AND METHODS

Strains and plasmids. E. coli K-12 strain MG1655ΔrelA was constructed by deleting the open reading frame of relA in the genomic DNA of MG1655 ΔrpoD-35 (14) using the procedure described by Datsonko and Wanner (6). PCR primers used were 5′-relA-D (5′-ATGTTGCGTAAGAAGTCTGAAGCCTTCTTCCATTTGCAAAAGCTG) and 3′-relA-D (CT AACCTCGTGACACCGCCTCGTGCTAAAATCATCCGACATTCGTTAATCTACCAGGGTA) and pKD13 (6) was used as a template. All plasmids were maintained and expressed in E. coli DH5α [supE44 thi-1 recA1 endA1 gyrB thi-1 (ΔmetE2 argF)] (C600 relA1). It was necessary to identify the genotype of DH5α, since the genomic library was constructed using genomic DNA isolated from DH5α whose relA genotype has not yet been determined. The genotype of relA is not defined, probably because one of the ancestors of DH5 was relA1, although the existence of this genotype has not been confirmed in DH5 (11). To verify the linkage of relA and the existence of relA in DH5, three of the DH5 ancestors (KL161 Hfr, KL16-99 Hfr, and Hfr7-4), which were genetically crossed to create another direct ancestor (DH2) of DH5, were tested for the presence of relA by amplifying the coding region of RelA in the context of an IS2 insertion that created relA1. The results clearly showed that only KL161 Hfr, one of the ancestors of DH5, carried relA1. The other ancestors and DH5 exhibit wild-type relA (data not shown). E. coli strains KL161 Hfr, KL16-99 Hfr, and Hfr7-4 were obtained from the E. coli Genetic Stock Center (Yale University).

Cultures were maintained in LB medium, and 100 μg/ml of ampicillin and 50 μg/ml of kanamycin were used as necessary. To induce the synthesis of plasmid-directed RNA from a T7/SS promoter, the RNA polymerase-I B-galactosidase (β-gal) promoter was added to a final concentration of 1 mM.

The coding region of RelA was amplified using two primers, RelA-S (5′-AG AATTCATATGTTGCCAGTAGCATG) and RelA-T (5′-TCTAGATCTA ACTCCCGTGCAACCCG-14) (14). Amplified DNA was digested with BglII and NdeI and ligated into the same sites in pKAN6B (42) to produce pKAN6A. Plasmid pKAN6 is a derivative of pKAN3, which contains an additional 3′-untranslated region. Plasmid pKAN7 is a derivative of pKAN3 (41) using E. coli genomic DNA from DH5α partially digested with BamHI. This plasmid was a derivative of pACYC177 and is compatible with the pRNA122 plasmid, which is a derivative of pBR322. The genomic library was constructed in pKAN3 (41) using E. coli genomic DNA from DH5α partially digested with BamHI. This plasmid was a derivative of pACYC177 and is compatible with the pRNA122 plasmid, which is a derivative of pBR322. The genomic library was constructed in E. coli cells that harbor pRNA122 with the U791 mutation (pRNA122-U791). The U791 mutation was chosen to select appropriate clones from the genomic library, since of possible mutations at this position, this mutation was known to be most detrimental to ribosome function, which resulted in a decrease in the protein synthesis ability of the mutant ribosomes to 4% without affecting the formation of the
30S ribosomal subunit (32). To select for genomic library clones containing the genes that, when overproduced, restored protein synthesis ability in U791 ribosomes, the cells expressing the U791 ribosomes from pRNA122-U791 were transformed with genomic library clones and plated on LB agar containing 50 \( \mu \text{g} \) of chloramphenicol (Cm) per ml of LB, at which cells expressing pRNA122-U791 ribosomes could not grow. Approximately 1 of every 500 transformants survived under this growth condition (205 survivors of 105 transformants), whereas only 1 of about 10^5 transformants survived when cells expressing pRNA122-U791 ribosomes were transformed with an empty vector (pKAN3) and selected under the same condition.

Next, to test if the isolated genomic library clones were responsible for the Cm resistance phenotype, plasmids were separately prepared from these clones and cotransformed with pRNA122-U791 into E. coli MG1655. The plasmids were separately prepared from 50 of the surviving genomic clones. To examine if overexpression of RelA was responsible for the partial restoration of protein synthesis by the pRNA122-U791 ribosomes, the coding region of relA was subcloned into pKAN6B (41, 42), a derivative of pKAN3, and expressed under control of the arabinos-inducible promoter (pRelA100), in order to test the degree of complementation to pRNA122-U791 ribosomes. Cells that expressed the pRNA122-U791 ribosomes and the RelA protein from pRelA100 showed resistance to Cm at the same levels (MIC = 125 \( \mu \text{g} \)/ml) as cells that expressed the pRNA122-U791 ribosomes and the RelA protein from the BamHI genomic clones (Table 1). Overexpression of RelA also allowed cells that expressed pRNA122-A791 or -C791 ribosomes to exhibit resistance to higher concentrations of Cm (MIC = 400 and 150 \( \mu \text{g} \)/ml, respectively), whereas the degree of Cm resistance of cells expressing the wild-type pRNA122 ribosomes was not affected by RelA overexpression (Table 1). When pRNA122-U791 ribosomes were expressed in E. coli cells that were deficient for relA, the degree of resistance to Cm was decreased compared to that of the isogenic relA strain that expressed the pRNA122-U791 ribosomes (Table 1). These results confirmed that the phenotypic changes were due to the gene dosage effects of relA.

Effects of RelA overproduction on CAT protein synthesis by pRNA122-U791 ribosomes. In an effort to confirm that the enhanced resistance to Cm in cells coexpressing the pRNA122-U791 ribosomes and RelA was a result of increased CAT protein synthesis by the mutant ribosomes, we quantified by Western blot analysis the amount of CAT protein in cells in the presence and absence of RelA overexpression. Cells expressing both pRNA122-U791 ribosomes and RelA showed an ~1.5-fold-increased amount of CAT protein compared to cells that expressed only the pRNA122-U791 ribosomes (Fig. 1). This showed a good correlation between the degree of cellular resistance to Cm and the quantity of CAT synthesized in these

**TABLE 1. Effects of relA gene dosage on Cm resistance of E. coli cells expressing the pRNA122 ribosome with nucleotide substitutions at position 791**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Residue in pRNA122 ribosome</th>
<th>Overexpression of RelA</th>
<th>Cm MIC (( \mu \text{g/ml} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH5(\alpha)</td>
<td>G791</td>
<td>–</td>
<td>600</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>600</td>
<td></td>
</tr>
<tr>
<td>A791</td>
<td>–</td>
<td>+</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>400</td>
<td></td>
</tr>
<tr>
<td>C791</td>
<td>–</td>
<td>+</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>150</td>
<td></td>
</tr>
<tr>
<td>U791</td>
<td>–</td>
<td>+</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>125</td>
<td></td>
</tr>
<tr>
<td>MG1655</td>
<td>U791</td>
<td>–</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>125</td>
<td></td>
</tr>
<tr>
<td>MG1655 ΔrelA</td>
<td>U791</td>
<td>–</td>
<td>50</td>
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<tr>
<td></td>
<td>+</td>
<td>100</td>
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305 ribosomal subunit (32). To select for genomic library clones containing the genes that, when overproduced, restored protein synthesis ability in U791 ribosomes, the cells expressing U791 ribosomes from pRNA122-U791 were transformed with genomic library clones and plated on LB agar containing 50 \( \mu \text{g} \) of chloramphenicol (Cm) per ml of LB, at which cells expressing pRNA122-U791 ribosomes could not grow. Approximately 1 of every 500 transformants survived under this growth condition (205 survivors of 105 transformants), whereas only 1 of about 10^5 transformants survived when cells expressing pRNA122-U791 ribosomes were transformed with an empty vector (pKAN3) and selected under the same condition.

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**FIG. 1. Effects of overexpression of RelA on the level of CAT protein production by mutant ribosomes.** Cultures were grown to an OD\(\text{600}\) of 0.1, and 1 mM IPTG and 0.1% arabinose were added to induce the synthesis of pRNA122 ribosomes and RelA. Culture samples were harvested 2 h after induction to obtain total protein. One milliliter of the culture at an OD\(\text{600}\) of 0.7 was harvested and resuspended in 80 \(\mu\)l of sodium dodecyl sulfate loading buffer. A 10-\(\mu\)l portion of the sample was then loaded in each lane. The same membrane was cut and probed with polyclonal antibodies to CAT and RelA. The relative abundance of the protein bands was quantified by setting the amount of protein produced by wild-type pRNA122 ribosomes in the absence of RelA overexpression to 1. The experiments were repeated three times, and the results were averaged. Means ± standard errors of the means indicate the range of the assay results.
cells. The amount of RelA protein in cells harboring pRelA100 was increased approximately 170-fold, compared to that in cells harboring pKAN6B (Fig. 1). This indicated that overexpression of RelA correlates with increased protein synthesis function of pRNA122-U791 ribosomes. Considering that the intracellular concentration of RelA was estimated to be about one copy per 200 ribosomes in E. coli cells grown under normal conditions (27), we estimated that the expression of the relA gene from pRelA100 yielded RelA protein levels similar to the number of ribosomes in the cell. Overexpression of RelA also allowed the synthesis of approximately 1.5 times more CAT protein by mutant ribosomes that contain A791 or C791. At the same time, the amount of CAT protein produced by the wild-type pRNA122 ribosomes was not significantly affected by the overexpression of RelA. Relative amounts of S1 were decreased by approximately 10 to 20% in cells that overexpressed RelA when the same OD600 units of cells (87.5 μl of OD600 units) were loaded in each lane. The modest 10 to 20% decrease in the S1 level could be explained by the fact that one of the rpsL promoters, rpsAp1, was under stringent control (7, 17) and may have been activated by the elevated basal level of (p)ppGpp as a result of RelA overexpression (see below).

Effects of RelA overproduction on the level of (p)ppGpp in DH5α cells harboring pRNA122. E. coli cells that overexpressed RelA exhibited elevated (p)ppGpp levels when grown in amino-acid-rich medium and elicited the typical effects of elevated (p)ppGpp levels, including slowed growth (29, 30), although intracellular levels of (p)ppGpp governed by RelA are normally regulated by aminoacyl-tRNA availability (40). However, the effects of RelA overexpression on the cellular concentrations of (p)ppGpp and growth rate were shown to be variable in the E. coli strains tested (29, 30). For this reason, we verified the ability of RelA expressed from pRelA100 in DH5α cells to elicit the elevated (p)ppGpp levels by measuring both the abundance of (p)ppGpp and the effects of RelA overexpression on growth rate. As shown in Fig. 2A, cells that coexpressed RelA and pRNA122-U791 ribosomes showed approximately a threefold increase in doubling time compared to cells that expressed pRNA122-U791 ribosomes only (180 versus 60 min). When DH5α cells harboring pRelA100 were grown in a minimal medium containing the full complement of amino acids, the basal levels of (p)ppGpp were elevated from about 0 pmol/A600 unit to about 17 pmol/A600 unit (Fig. 2B). The (p)ppGpp levels in the DH5α strain are comparable to those measured in other E. coli strains grown in amino-acid-rich media (29, 30), although the simplified chromatography method that we used did not accurately measure the low baseline level of (p)ppGpp. While the (p)ppGpp levels were moderately increased when DH5α cells harboring pRelA100 were grown in an amino-acid-rich medium, the (p)ppGpp levels were dramatically increased to 122 pmol/A600 unit when amino acid starvation was triggered by adding serine hydroxamate to the cultures. When amino acid starvation was triggered, DH5α cells that harbored an empty vector (pKAN6B) also showed a significant increase in (p)ppGpp levels (~40 pmol/A600 unit), which were 2.4 times higher than the levels in RelA-overexpressing DH5α cells grown in an amino-acid-rich medium. These results indicated that the DH5α cells that overproduced RelA may not have accumulated sufficiently high (p)ppGpp levels to effectively trigger the stringent response in amino-acid-rich media. For this reason, the phenotype of slowed growth may have stemmed from an unknown effect of increased RelA levels, as has been previously suspected (30). Nonetheless, DH5α cells that overexpressed RelA produced more CAT proteins and consequently exhibited increased resistance to Cm, even though the growth rate was dramatically decreased.

Effects of RelA overproduction on the relative amounts of rRNA and CAT mRNA from pRNA122. Since the (p)ppGpp levels and the growth rate were altered in the cells when RelA was overexpressed, we thought that the mechanism of enhanced CAT protein synthesis by mutant ribosomes in the presence of increased RelA might be related to the increased ratio of mutant mRNA to the total rRNA pool. To test this possibility, we used a modified primer extension method (32) to determine the proportions of plasmid and chromosome-derived RNA in total ribosome pool. As shown in Fig. 3A, the ratio of the 27-nucleotide-long DNA fragments synthesized from plasmid-derived mutant rRNA to the 22-nucleotide-long DNA fragments synthesized from chromosome-derived unmethylated Cm remained unchanged in cells, regardless of the overexpression of RelA. This result suggests that the partial restoration of protein synthesis ability to mutant ribosomes, through increased RelA expression, was not the result of the increased ratio of mutant rRNA to total rRNA in the cell.

Since it was also possible that the enhanced CAT protein synthesis by mutant ribosomes in the presence of high RelA levels resulted from increased CAT mRNA levels, we also tested the effect of overexpression of RelA on CAT mRNA synthesis from pRNA122 by measuring the steady-state level of CAT mRNA using Northern blot analysis. We observed that the levels of CAT mRNA were decreased by ~40% when the amount of CAT mRNA was normalized to the steady-state level of 5S rRNA in RelA-overexpressing cells (Fig. 3B). The increased levels of CAT mRNA in the context of RelA overexpression may have resulted from the repression of a mutant tryptophan promoter (Ptpr) used for the constitutive synthesis of CAT mRNA (16) by the elevated levels of (p)ppGpp. However, it is more likely that a slowing of cellular growth by RelA overexpression indirectly resulted in decreased levels of CAT mRNA, since the tryptophan promoter is known to be insensitive to ppGpp levels. In any case, these results strongly indicate that the enhanced production of CAT protein by the pRNA122-U791 ribosome was not due to the increased steady-state level of CAT mRNA in RelA-overexpressing cells.

Effects of RelA overproduction on CAT protein synthesis by different types of ribosomes. We further tested the effect of RelA overexpression on wild-type ribosomes that contained unmethylated mRNA binding sequences, which were present in chromosomally derived 16S rRNA. We measured the amount of CAT protein produced by cells expressing CAT mRNA with a natural E. coli consensus ribosome binding sequence (5′-GAGG-3′) and 16S rRNA with an mRNA binding sequence (5′-CCUC-3′) present in chromosomally derived rRNA (pRNA9) (19) in the presence and absence of RelA overexpression. Our results showed that overexpression of RelA did not enhance the level of CAT protein synthesis by wild-type
ribosomes (Fig. 4), indicating that RelA overexpression did not enhance the protein synthesis function of ribosomes that bear a wild-type residue at position 791 (G791). This result held true regardless of the mRNA binding sequences (Fig. 1A).

In order to test whether the effects of increased RelA acting as a multicopy suppressor were specific to pRNA122-U791, DH5α cells harboring pRNA122-A516 were transformed with pKAN6 or pRelA100, and the resulting transformants were...
tested for the degree of resistance to Cm. The A516 mutation was chosen because pRNA122-A516 ribosomes had previously been shown to exhibit protein synthesis ability that was as poor as that of pRNA122-U791 ribosomes (21). Our results showed that RelA overexpression had no effect on pRNA122-A516 ribosomes, thus indicating that the effect of RelA on ribosome function was not a general phenomenon (data not shown).

Isolation of RelA mutants that exhibit an impaired ability to trigger the stringent response while complementing the pRNA122-U791 ribosome. To further show that the (p)ppGpp synthesis activity of RelA was not the basis for the partial enhancement of protein synthesis ability of pRNA122-U791 ribosomes, we searched for mutant RelA proteins whose overproduction did not result in the synthesis of sufficient (p)ppGpp and consequently did not trigger the stringent response but still complemented the mutant ribosome as effectively as wild-type RelA. To achieve this, pRelA100 plasmids bearing random mutations in the coding region of RelA were transformed into DH5α cells harboring pRNA122-U791 and pKAN6B at 1. The amount of CAT mRNA was normalized using the amount of 5S rRNA. The experiments were repeated three times, and results were averaged. Means ± standard errors of the means indicate the range of the assay results.

FIG. 3. Effects of RelA overexpression on the relative amounts of rRNA and CAT mRNA from pRNA122. (A) Effects of overexpression of RelA on the proportion of plasmid-derived rRNA in the total ribosome pool. A modified primer extension method was used to determine the percent plasmid-derived rRNA. The end-labeled primer complementary to the sequence between positions 793 and 812 in 16S rRNA was annealed to total rRNA purified from ribosomes and extended through the mutation site using reverse transcriptase. Results for the 27-nucleotide DNA fragments synthesized from plasmid-derived mutant rRNA and the 22-nucleotide DNA fragments synthesized from chromosome-derived wild-type rRNA are shown. In the first lane, samples from extension reactions carried out without RNA were loaded. (B) Effects of RelA overexpression on the steady-state level of CAT mRNA. The membrane that had been probed for CAT mRNA was stripped and reprobed for 5S rRNA. The relative abundance of CAT mRNA bands was quantified by setting the amount of mRNA produced from pRNA122 in the absence of RelA overproduction (DH5α cells harboring pRNA122-U791 and pKAN6B) at 1. The amount of CAT mRNA was normalized using the amount of 5S rRNA. The experiments were repeated three times, and results were averaged. Means ± standard errors of the means indicate the range of the assay results.

FIG. 4. Effects of RelA overexpression on wild-type ribosomes containing unmuted mRNA binding sequence. DH5α cells harboring pRNA122 and pKAN6B or pRelA100 were grown, and the amount of CAT protein was measured as described in the legend to Fig. 2B.
the amino acid substitutions in the mutant RelA proteins resulted in the altered stability of the protein (Fig. 5A). Based on these results, we felt that the ability of pRNA122-U791 ribosomes to synthesize more CAT protein was likely not due to the (p)ppGpp synthesis activity of the RelA protein.

Since sensitivity to AT may discriminate only between high and low levels of (p)ppGpp in cells, we chose to measure the accumulation of (p)ppGpp as a result of mutant RelA protein overexpression during amino acid starvation. To avoid higher (p)ppGpp levels in the \textit{relA}/H11001 strains under amino acid starvation conditions (Fig. 2), the plasmids expressing RelA(pRelA100) or mutant RelA proteins were transformed into the \textit{relA} strain MG1655/H9004, and the amount of (p)-ppGpp was measured during amino acid starvation. The degree of accumulation of (p)ppGpp in cells that overexpressed mutant RelA proteins ranged from 18.2 to 38.9% of the (p)-ppGpp levels in cells that overexpressed the wild-type form of RelA (Fig. 5B). We observed no correlation between the level of (p)ppGpp accumulation and the degree of resistance to Cm. Despite the huge differences in the degree of (p)ppGpp accumulation, the growth rate of DH5\textalpha{} cells that overexpressed mutant RelA proteins was similar to that of the cells expressing wild-type RelA (data not shown), indicating that it is unlikely to be high levels of (p)ppGpp accumulation that resulted in the slowed growth. This notion was further supported by the fact that DH5\textalpha{} cells harboring pRelA100 grown in amino acid-rich medium did not accumulate high levels of (p)ppGpp (~17 pmol/\text{A}_{600} unit), compared to DH5\textalpha{} cells harboring an empty vector (pKAN6B) grown under amino acid starvation (Fig. 2). Once again, these results indicated that the slow-growth phenotype of DH5\textalpha{} cells overexpressing RelA may have stemmed from an unknown effect of overexpressed RelA, rather than the stringent response triggered in amino acid-rich media.

**DISCUSSION**

The involvement of RelA in the complementation of mutant ribosomes was interesting in view of its functional role in bacterial ribosomes. It has been proposed elsewhere that the EF-Tu \cdot ppGpp complex increases the fidelity of proofreading in protein synthesis (9). However, to our knowledge, no evidence has been reported to date regarding the involvement of RelA in ribosome function. Our data provide evidence for RelA involvement in ribosome function and identify a possible role for the RelA protein, namely, that of changing a nonfunctional structure of the mutant 790 loop to a functional one, by directly or indirectly interacting with the loop. Overexpression of RelA partially enhanced the protein synthesis ability of mutant ribosomes that bear a base substitution at position 791. Our experiments with the mutant variants of RelA that were defective in (p)ppGpp synthetic activity, but not in the suppression of the G791U mutation, indicated that the (p)ppGpp synthetic activity did not determine the suppressor ability of RelA. However, the effect of RelA was shown to be specific to residue 791, since we observed no effects on the activity of either wild-type ribosomes or specialized ribosomes containing a wild-type loop 790 or bearing A516 (another harmful mutation). Thus, it was
not obvious that RelA has any other functions (besides its role in stringent response) in the normal translation process by wild-type ribosomes.

The exact parameters that modulate the efficiency of RelA binding to ribosomes as well as (p)ppGpp synthesis have not been adequately characterized to date. However, it has been shown that RelA is associated with the 30S subunit of the ribosome (28), and the N terminus of ribosomal protein L11 is involved in the regulation of RelA activity, although direct interaction between L11 and RelA has not been demonstrated (39). L11 forms a complex with a segment (nucleotides 1051 to 1108) of 23S rRNA (37) that overlaps with the binding sites of the elongation factors, EF-Tu and EF-G. Based on this observation, Wimberly et al. (37) proposed that the N-terminal domain of L11 may represent a molecular switch that selects between RNA-bound and RNA-free states and functions by regulating the conformation or the accessibility of the RNA in the GTPase-associated site during the elongation cycle of protein synthesis. The data showing the placement of the 790 loop at the interface of the 30S subunit, where it directly interacts with components of the 50S subunit in the decoding process, together with the enhanced binding of RelA to the ribosome on account of the presence of mRNA (36), imply the existence of a network that links RelA ribosome binding with the decoding process. Based on our data and the results of other groups, it is tempting to speculate on an involvement of RelA in restoring the structural perturbation caused by a base substitution at position 791 by activation of the L11 N-terminal switch domain that is probably promoted by increased binding of RelA to the ribosome.

It is unlikely that the elevated levels of (p)ppGpp can directly or indirectly affect pRNA122-U791 ribosome function, since we did not observe a correlation between the degree of (p)ppGpp accumulation in the context of mutant RelA proteins and the amount of CAT protein synthesized by the pRNA122-U791 ribosomes. In the future, it would be valuable to attempt a structural elucidation of the ribosome-RelA complex, in order to identify more detailed molecular mechanisms of the action of RelA in the context of translation.

ACKNOWLEDGMENTS

We thank Edward E. Ishiguro for providing us with monoclonal antibodies to RelA.

This work was supported by grants from the Korean Research Foundation (KRF-2006-311-C00536) and the 21C Frontier Microbial Genomics and Application Center Program of the Ministry of Education, Science & Technology (Republic of Korea) to K. Lee.

REFERENCES


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