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

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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 $tRNA^{Phe}$ (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 (ppGpp), which are collectively referred to as (p)ppGpp (13, 34). The synthesis reaction occurs when the binding of uncharged tRNA to the aminoacyl-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-

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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 (*ilvG rfb-50 rph-1*) (1) using the procedure described by Datsenko and Wanner (6). PCR primers used were 5′-relA-D (5′-ATGGTTGCGGTAAGAAGTGCACATATCAATAAG GCTGGTGAATTTGATGTGTAGGCTGGAGCTGCTTC) and 3′-relA-D (CT AACTCCCGTGCAACCGACGCGCGTCGATAACATCCGGCACCTGGTTA TTCCGGGGATCCGTCGACC), and pKD13 (6) was used as a template. All plasmids were maintained and expressed in *E. coli* DH5α [*supE44 hsdR17 recA1 endA1 gyrA96 thi-1* Δ(*lacIZYA argF*) *U169 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 lineage of *relA1* 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 added as necessary. To induce the synthesis of plasmid derived rRNA from the *lacUV5* promoter, IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 1 mM.

The coding region of RelA was amplified using two primers, RelA-S (5′-AG AATTCATATGGTTGCGGTAAGCAGT) and RelA-T (5′-ATCTAGATCTA ACTCCCGTGCAACCG) (14). Amplified DNA was digested with BglII and NdeI and ligated into the same sites in pKAN6B (42) to produce pRelA100. Plasmid pKAN6 is a derivative of pKAN3, which contains an additional *araC* gene and an arabinose-inducible promoter (P_{BAD}). To randomly introduce base substitutions into the coding region of RelA in pRelA100, the DNA segment encoding RelA was amplified in an error-prone PCR mix that additionally contained 0.1 mM $MnCl_2$ (31). The reaction products were then cloned into pKAN6B as described above. PCR primers used were RelA-S and RelA-T. A DNA segment encompassing an IS*2* insertion in *relA1* was amplified using the primers RelA-514F (5′-TACTGGCGGCAAAAGAG) and RelA-1270R (5′-GC GTTGATCCCGCAGGC). Plasmid pKAN6B is a derivative of pKAN6 (41) that contains a unique BglII site for cloning BamHI-digested chromosomal DNA. Plasmid pKAN6B was constructed by ligating the BglII- and NdeI-digested pKAN6-IF1 (42) DNA fragment after filling in the ends with a Klenow fragment of DNA polymerase I.

MICs. MICs were determined as previously reported (19).

Protein and RNA work. Cultures were grown to an optical density at 600 nm (OD_{600}) of 0.1. IPTG (1 mM) was added to induce the synthesis of pRNA122-ribosomes, and 0.1% arabinose was further added to induce the synthesis of RelA from pRelA100. Culture samples were harvested 2 h after induction to obtain crude ribosomes, total protein, or RNA. Purification of crude ribosomes has been previously described (32). The relative abundance of protein bands was quantified using the Versa Doc imaging system (Bio-Rad) and the Quantity One software package (v. 4.5.1; Bio-Rad). The percents plasmid- and chromosome-derived rRNA in whole ribosomes were determined by a modified primer extension (32) by annealing the end-labeled primer 16S-812R (5′-CGGCGTGGA CTACCAGGGTA) complementary to the 791 mutation site and extending through the mutation site using avian myeloblastosis virus reverse transcriptase. The extension reaction mixture contained three kinds of deoxynucleotides (dATP, dGTP, dTTP) and one kind of dideoxynucleotide (ddCTP). The synthesized cDNAs were resolved by polyacrylamide gel electrophoresis, and the ratios of mutant to nonmutant rRNA were determined by comparing the amounts of radioactivity in each of the two bands. The procedure for Northern blot analysis has been described previously (41) and was performed using 5′-end labeled oligonucleotides. The 5S probe I (5′-ACTACCATCGGCGCTAC GGC) for probing 5S rRNA and the probe Cat + 90 (5′-GCAACTGACTG AAATGCCTC) for probing chloramphenicol acetyltransferase (CAT) mRNA were used.

Measurements of (p)ppGpp accumulation. Cells were grown in MOPS (morpholinepropanesulfonic acid) minimal medium containing 1.32 mM phosphate and supplemented with 0.2% glucose and all amino acids. Overnight cultures were diluted in MOPS minimal medium containing 0.2 mM phosphate, 0.01% glucose, and all amino acids. For cultures under the condition of amino acid starvation, cells were cultured without serine. At an OD_{600} of 0.1, the cells were uniformly labeled with $^{32}P_i$. We then performed plasmid induction by adding 0.1 mM IPTG and 0.1% arabinose. At an OD_{600} of 0.2, amino acid starvation was induced by adding 1 mg of serine hydroxamate/ml into the cultures that had been grown in the absence of serine. Samples were withdrawn at various intervals and analyzed for their (p)ppGpp content by thin-layer chromatography as described elsewhere (10, 30).

RESULTS

Isolation of multicopy suppressors for pRNA122-U791 ribosome. A previous study that utilized a novel procedure termed instant evolution was able to identify G791 in the SSU rRNA as an invariant residue for ribosome function (20). This procedure adopted a specialized ribosome system whereby the CAT reporter message was exclusively translated by the plasmid (pRNA122)-derived ribosomes (pRNA122 ribosomes) that could not translate normal cellular message (19–21). Thus, it was possible to assay the functions of the plasmid-derived mutant ribosomes in vivo by determining the MICs of cells that express the mutant ribosomes. Using the same specialized ribosome system, we subsequently showed that a base substitution at position 791 in *E. coli* 16S rRNA resulted in attenuation of the ability of ribosomes to conduct protein synthesis by more than 65% (32).

We speculated that a base substitution at position 791 may cause a structural perturbation in the 790 loop that prevents residue 791, or the loop itself, from interacting with a ligand involved in protein synthesis. Thus, overproduction of this ligand may restore protein synthesis function to the mutant ribosome. In order to identify such a ligand, we used a genetic complementation approach. A genomic library was constructed in pKAN3 (41) using *E. coli* genomic DNA from DH5α partially digested with BamHI. This plasmid is a derivative of pACYC177 and is compatible with the pRNA122 plasmid, which is a derivative of pBR322. The genomic library was transformed into *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

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

Strain	Residue in pRNA122 ribosome	Overexpression of RelA	Cm MIC (μg/ml)
DH5α	G791	−	600
		+	600
	A791	−	200
		+	400
	C791	−	75
		+	150
	U791	−	50
		+	125
MG1655	U791	−	75
		+	125
MG1655 ΔrelA	U791	−	50
		+	100

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 μg 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 10^5 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 50 of the surviving genomic clones. The plasmids were separately prepared from these clones and cotransformed with pRNA122-U791 into *E. coli* cells. The resulting transformants were tested for their degree of resistance to Cm in the presence and absence of the inducer IPTG. All clones were resistant to 100 μg/ml Cm (MIC = 125 μg/ml) only in the presence of IPTG, indicating that CAT mRNA translation in these cells did depend on both the pRNA122-U791 ribosomes and the genomic clones. Ten of the clones derived from cells harboring an empty vector were subjected to the procedure described above. This resulted in a loss of resistance to 100 μg/ml Cm, indicating that these clones probably carried chromosomal mutation(s).

To determine if the isolated clones shared a common region of the *E. coli* chromosome, we performed restriction enzyme sites analyses with plasmids purified from the 50 clones using the initial BamHI cloning site. All the clones that offered enhanced resistance to Cm (MIC = 125 μg/ml) exhibited a common 6.5-kbp BamHI fragment. We subsequently sequenced the chromosomal DNA in two clones that contained only the common 6.5-kbp BamHI fragment. The results showed that the fragment was located at 62 min of the *E. coli* chromosome, which contained the coding region of *relA*. To examine if overexpression of RelA was responsible for the partial restoration of protein synthesis function by the pRNA122-U791 ribosomes, the coding region of *relA* was subcloned into pKAN6B (41, 42), a derivative of pKAN3, and expressed under the control of the arabinose-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 μg/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 μg/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

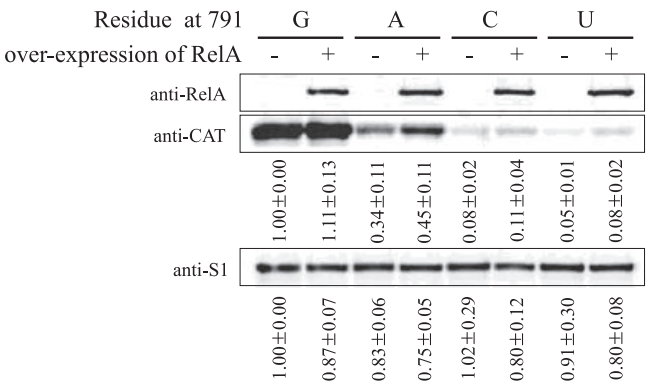

FIG. 1. Effects of overexpression of RelA on the level of CAT protein production by mutant ribosomes. Cultures were grown to an OD_{600} of 0.1, and 1 mM IPTG and 0.1% arabinose were added to induce the synthesis of pRNA122 ribosomes and RelA. Culture samples were harvested 2 h after induction to obtain total protein. One milliliter of the culture at an OD_{600} of 0.7 was harvested and resuspended in 80 μl of sodium dodecyl sulfate loading buffer. A 10-μl portion of the sample was then loaded in each lane. The same membrane was cut and probed with polyclonal antibodies to CAT and RelA and with polyclonal antibodies to S1. 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 OD_{600} units of cells (87.5 μl of OD_{600} 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 *rpsA* 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/A_{600} unit to about 17 pmol/A_{600} 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/A_{600} 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/A_{600} 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 rRNA 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 rRNA 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 unmutated rRNA 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 decreased levels of CAT mRNA in the context of RelA overexpression may have resulted from the repression of a mutant tryptophan promoter ($Ptrp^c$) 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 unmutated 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′-GGAGG-3′) and 16S rRNA with an mRNA binding sequence (5′-CCUCC-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

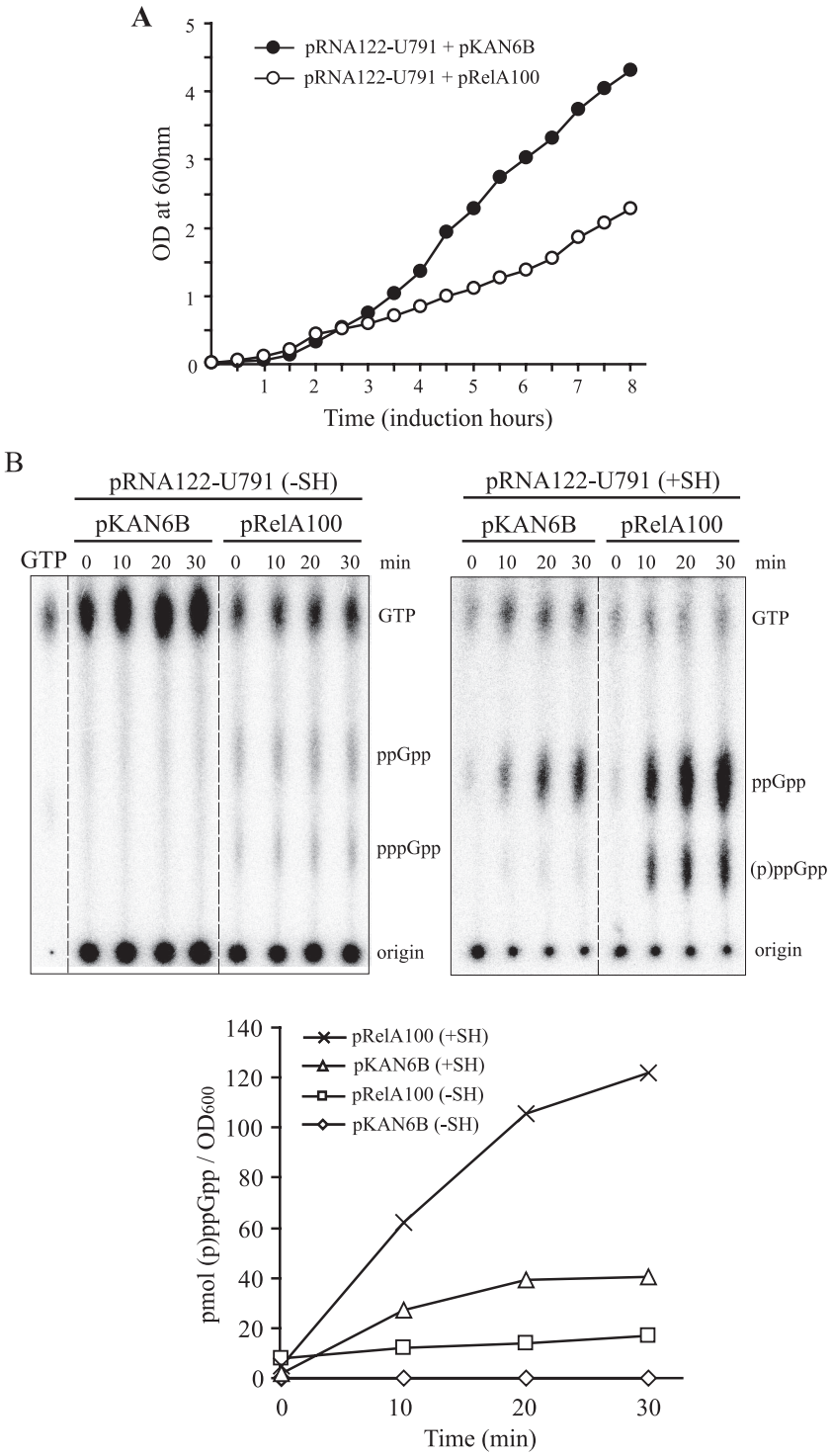

FIG. 2. Effects of RelA overexpression on the physiology of DH5α cells harboring pRNA122. (A) Effects of overexpressing RelA on growth. *E. coli* DH5α cells harboring pRNA122-U791 and pKAN6B or pRelA100 were grown in LB containing ampicillin (100 μg/ml) and kanamycin (50 μg/ml) and monitored for growth rate at 600 nm. At an OD_{600} of 0.1, 1 mM IPTG and 0.1% arabinose were added to the cultures, and the absorbance at 600 nm was measured at the times indicated. (B) Effects of overproduction of RelA on cellular (p)ppGpp accumulation. *E. coli* DH5α cells harboring pRNA122-U791 and pKAN6B or pRelA100 were grown in MOPS minimal medium and analyzed for their (p)ppGpp content by thin-layer chromatography (see Materials and Methods). SH, serine hydroxamate.

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

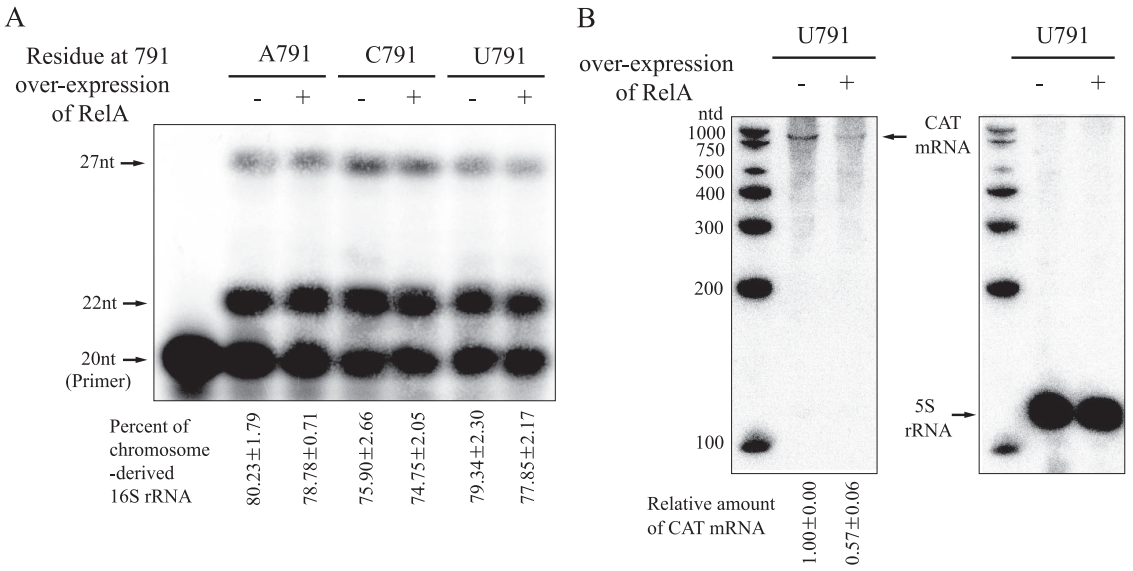

FIG. 3. Effects of RelA overexpression on the relative amounts of rRNA and CAT mRNA from pRNA122. (A) Effects of overexpression of RelA on the proportion of plasmid-derived rRNA in the total ribosome pool. A modified primer extension method was used to determine the percent plasmid-derived rRNA. The 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.

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. The transformed cells were selected on LB agar medium containing 100 μg/ml Cm, where cells that harbor pRNA122-U791 and pKAN6B cannot grow. The transformants that survived on LB agar medium containing 100 μg/ml Cm were individually tested for their ability to grow in the presence of 15 mM AT, a histidine analog that induces histidine starvation (15). This screening method took advantage of the fact that *E. coli* cells can grow in the presence of AT only if the cellular levels of (p)ppGpp are sufficiently high to positively regulate the *his* operon (33). Thirty-seven of 612 transformants showed a loss of ability to grow in the presence of 15 mM AT. The coding region of *relA* in pRelA100 plasmid from the three clones was then sequenced to identify mutations. The sequencing analysis showed that the clones carried mutations that resulted in one to four amino acid substitutions in RelA (Fig. 5A). Cells harboring pRNA122-U791 and overexpressing the mutant RelA proteins showed the same levels of Cm resistance as cells that overexpressed the wild-type RelA (Fig. 5A). Western blot analysis showed that the steady-state levels of mutant RelA proteins ranged from 38 to 125% of the levels found in cells that overexpressed the wild-type form of RelA This indicated to us that

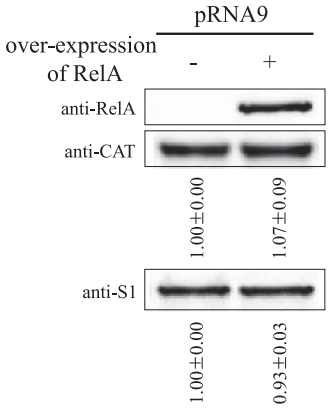

FIG. 4. Effects of RelA overexpression on CAT protein synthesis by wild-type ribosomes containing unmutated mRNA binding sequence. DH5α cells harboring pRNA122 and pKAN6B or pRelA100 were grown, and the amount of CAT protein was measured as described in the legend to Fig. 2B.

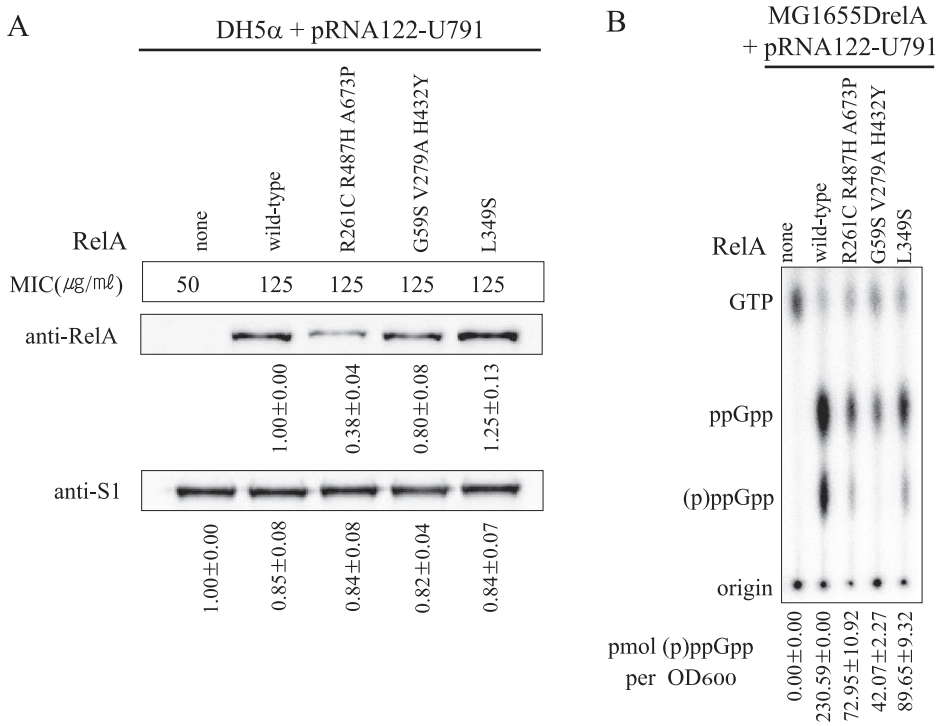

FIG. 5. Characterization of isolated RelA mutants. (A) Expression levels of mutant RelA proteins in DH5α cells that coexpressed pRNA122-U791 ribosomes. The amounts of the wild-type and mutant RelA proteins were measured as described in the legend to Fig. 1. (B) (p)ppGpp synthetic activity of mutant RelA proteins. Strain MG1633ΔrelA was transformed with pKAN6B, pRelA100, or pRelA100 mutants, grown in MOPS minimal medium, and analyzed for (p)ppGpp content by thin-layer chromatography (see Materials and Methods).

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 *relA*$^+$ strains under amino acid starvation conditions (Fig. 2), the plasmids expressing RelA (pRelA100) or mutant RelA proteins were transformed into the *relA* strain MG1655ΔrelA, 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 DH5α 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 DH5α cells harboring pRelA100 grown in amino acid-rich medium did not accumulate high levels of (p)ppGpp (~17 pmol/A_{600} unit), compared to DH5α cells harboring an empty vector (pKAN6B) grown under amino acid starvation (Fig. 2). Once again, these results indicated that the slow-growth phenotype of DH5α 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 · 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 50S 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.

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