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Roles of Transcriptional and Translational Control Mechanisms in Regulation of Ribosomal Protein Synthesis in *Escherichia coli*

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ABSTRACT Bacterial ribosome biogenesis is tightly regulated to match nutritional conditions and to prevent formation of defective ribosomal particles. In *Escherichia coli*, most ribosomal protein (r-protein) synthesis is coordinated with rRNA synthesis by a translational feedback mechanism: when r-proteins exceed rRNAs, specific r-proteins bind to their own mRNAs and inhibit expression of the operon. It was recently discovered that the second messenger nucleotide guanosine tetra and pentaphosphate (ppGpp), which directly regulates rRNA promoters, is also capable of regulating many r-protein promoters. To examine the relative contributions of the translational and transcriptional control mechanisms to the regulation of r-protein synthesis, we devised a reporter system that enabled us to genetically separate the *cis*-acting sequences responsible for the two mechanisms and to quantify their relative contributions to regulation under the same conditions. We show that the synthesis of r-proteins from the S20 and S10 operons is regulated by ppGpp following shifts in nutritional conditions, but most of the effect of ppGpp required the 5' region of the r-protein mRNA containing the target site for translational feedback regulation and not the promoter. These results suggest that most regulation of the S20 and S10 operons by ppGpp following nutritional shifts is indirect and occurs in response to changes in rRNA synthesis. In contrast, we found that the promoters for the S20 operon were regulated during outgrowth, likely in response to increasing nucleoside triphosphate (NTP) levels. Thus, r-protein synthesis is dynamic, with different mechanisms acting at different times.

IMPORTANCE Bacterial cells have evolved complex and seemingly redundant strategies to regulate many high-energy-consuming processes. In *E. coli*, synthesis of ribosomal components is tightly regulated with respect to nutritional conditions by mechanisms that act at both the transcription and translation steps. In this work, we conclude that NTP and ppGpp concentrations can regulate synthesis of ribosomal proteins, but most of the effect of ppGpp is indirect as a consequence of translational feedback in response to changes in rRNA levels. Our results illustrate how effects of seemingly redundant regulatory mechanisms can be separated in time and that even when multiple mechanisms act concurrently their contributions are not necessarily equivalent.

KEYWORDS ribosome synthesis, translational feedback regulation, autogenous control, stringent response, ppGpp, regulation of promoter activity

The ribosome is one of the largest consumers of cellular resources during fast growth, and thus its biosynthesis must be regulated appropriately with respect to nutritional conditions. This represents a unique challenge, as the cell has to synthesize three rRNA molecules and more than 50 different ribosomal proteins stoichiometrically and in ample quantities to support cell growth while simultaneously preventing

wastage of resources and formation of defective ribosome particles (1, 2). In *Escherichia coli*, decades of research have shown that the synthesis of most ribosomal proteins (r-proteins) is regulated by feedback mechanisms in which specific r-proteins act as translational repressors when they accumulate in excess of rRNA, binding to their own mRNAs to inhibit synthesis of r-proteins from their respective operons (reviewed in references 1 and 3). Since binding of the repressor is to a single site on each mRNA, additional mechanisms, such as translational coupling, retroregulation, and transcription attenuation, are thought to account for regulation of other genes within polycistronic operons (1, 3). The r-protein repressors have a higher affinity for rRNA than for their mRNA targets, ensuring that when free rRNA is available, ribosome assembly is favored over inhibition of r-protein synthesis, making rRNA the rate-limiting substrate for ribosome synthesis (1).

rRNA transcription is tightly regulated to balance ribosome synthesis with the cellular requirement for protein synthesis (4, 5). The cell adjusts rRNA synthesis in large part by using nucleoside triphosphate (NTP) concentrations and guanosine tetra- and pentaphosphate (ppGpp) (for brevity, both the tetra- and pentaphosphate versions are referred to here as ppGpp) as signals of the nutritional state of the cell (4). For example, intracellular ppGpp levels increase dramatically in response to amino acid starvation, which results in a sharp inhibition of ribosome synthesis and an increase in amino acid synthesis, referred to as the stringent response (6). ppGpp and NTP concentrations regulate rRNA synthesis primarily at the level of transcription initiation (reviewed in reference 5). rRNA promoters have evolved with intrinsic kinetic properties that result in a requirement for higher initiating NTP concentrations than most other promoters, as well as a high sensitivity to inhibition by ppGpp (4, 7, 8). ppGpp regulates transcription initiation by binding directly to two separate sites on RNA polymerase (RNAP) (9, 10). The RNAP binding factor, DksA, which contributes to the formation of one of the ppGpp binding sites on RNAP (10), also increases the dependence of rRNA promoters on the initiating NTP concentration and acts synergistically with ppGpp to inhibit transcription initiation from these promoters (11, 12). Therefore, in contrast to most other promoters, rRNA promoters are strongly inhibited when NTP concentrations are low and/or when ppGpp concentrations are high, such as when cells are starved for nutrients or during the transition to stationary phase (4, 8, 11).

Direct transcriptional regulation of rRNA synthesis with respect to nutritional conditions, together with indirect regulation of r-protein synthesis through translational feedback, is theoretically sufficient to explain both the balanced synthesis of every ribosomal component and the coordination of ribosome synthesis with nutritional conditions (1, 5). However, early work indicated that transcription of various r-proteins was inhibited by ppGpp *in vitro* (13), the *rpsJ* (S10) r-protein promoter was stringently controlled (14), and ppGpp inhibited transcription of the *rpsT* (S20) gene in a coupled transcription-translation system *in vitro* (15), suggesting that at least some r-protein promoters are regulated by ppGpp. Nonetheless, in many cases, it was shown that translational feedback accounted for stringent control of r-protein operons, e.g., in the L11, *spc*, and α r-protein operons (16, 17). Thus, when early global analyses showed that transcript levels of many r-protein operons were reduced during amino acid starvation (18), the results were attributed to indirect effects on r-protein mRNA levels caused by translational feedback inhibition in response to ppGpp directly regulating rRNA synthesis. More recently, we reported that many r-protein promoters were regulated directly by ppGpp and DksA (19), suggesting that there may be redundancy in ppGpp-dependent regulation of r-protein synthesis. It was also reported recently that the *rplM-rpsI*, *rpmB-rpmG*, and *rplU-rpmA* operons were regulated at the transcription level by ppGpp and DksA, but only *rplM-rpsI* was regulated at the translation level as well (20).

To evaluate the relative impact of the transcriptional and translational control mechanisms on r-protein regulation by ppGpp, we reexamined the regulation of two r-protein operons whose promoters were strongly regulated by ppGpp in the previous study (19), the *rpsT* (S20) operon and the *rpsJ* (S10) operon, both of which were also

documented as being regulated by translational feedback (3). We show here that most regulation of the S20 operon following induction of ppGpp is indirect and likely through the translational feedback mechanism, whereas the promoters of the S20 operon are regulated during outgrowth, likely in response to increased NTP levels. Likewise, most regulation of the *rpsJ* operon by ppGpp is also promoter independent. We propose that posttranscription initiation events are the main targets for regulators of r-protein synthesis in response to changes in nutritional conditions during exponential growth, whereas regulation of r-protein promoter activity by NTPs is important for preventing expression during stationary phase and for quickly restarting ribosome synthesis during outgrowth from stationary phase. Thus, regulation of r-protein synthesis is dynamic, with different mechanisms playing roles at different times.

RESULTS

Design of a reporter system for measuring S20 synthesis. Although the sequences required for translational feedback control of the *rpsT* mRNA were not defined exhaustively and direct binding of S20 to the *rpsT* mRNA was never demonstrated (21), there is substantial literature regarding autoregulation of S20 synthesis at the level of translation (reviewed in reference 3). Gene dosage experiments showed that S20 expression is regulated at a posttranscriptional step (22), coupled transcription-translation experiments showed that S20 inhibits its own synthesis directly from a DNA template carrying the S20 structural gene (23, 24), and *in vitro* translation experiments showed that excess 16S rRNA results in increased S20 synthesis (15), presumably from derepression of translational feedback. Furthermore, overexpression of S20 resulted in inhibition of expression from an *rpsT* leader/ribosome binding site (RBS)-*lacZ* fusion (25), chemical and enzymatic probing showed that the *rpsT* mRNA segment encompassing the first 18 codons of S20 folds into a structure containing two hairpins (26), and mutation of the UUG start codon to AUG eliminated autoregulation (25). It was therefore proposed that the hairpins are part of the regulatory target for S20 and, along with the ribosome binding site, constitute the sequences needed for translational feedback inhibition (26).

Based on this information, we designed a reporter system to detect effects of ppGpp on the *rpsT* promoters separately from effects on translation of S20. Schematics of the reporters are shown in Fig. 1A (details in legend). To create open reading frames (ORFs) of the same size for each construct, the reporters contained the N-terminal 18 codons of the S20 gene (to include the two hairpins in the mRNA described above) fused in frame with the coding sequence for the streptavidin-binding peptide (SBP) (27) and the gene for β-galactosidase (*lacZ*). The SBP tag was included to increase the size of the reporter protein slightly, resulting in its migration to a position devoid of other proteins when visualized in a discontinuous SDS-PAGE system (28) (Fig. 1B).

The reporters contained either the *rpsT* promoter(s) or the *lacUV5* promoter, as well as the leader region from either *rpsT* or *lacZ*. We use "*rpsT* leader" to refer to the *rpsT* mRNA region that starts immediately downstream of *rpsT* P2 and contains the first 18 codons of S20 fused to SBP-*lacZ* (Fig. 1A, reporters 1, 3, 5). The "*lacZ* leader" constructs (reporters 2, 4, 6, 7, 8) have the *lacZ* mRNA leader region and the first 18 codons of S20 fused to SBP-*lacZ* but with an AUG start codon instead of UUG (25) as well as multiple silent mutations in the S20 (1 to 18) coding sequence to further ensure loss of regulation via translational feedback (MUT S20 [1 to 18]) (details in the supplemental material). The remaining reporters (9, 10) are control constructs described below.

Since the reporter protein is too stable to measure rapid changes in synthesis rates by β-galactosidase activity assays, we instead measured synthesis by pulse-labeling with L-[^{35}S]methionine. To verify that the reporter protein band originated from the corresponding SBP-*lacZ* fusion, we compared its migration to that from a construct that encoded a smaller, SBP-less version of the reporter protein (Fig. 1A and B, reporters and lanes 9 and 10, respectively). A promoterless reporter fusion and a strain without a reporter fusion did not show significant synthesis of the reporter protein (Fig. 1B, lanes 11 and 12, respectively), indicating that background levels of reporter synthesis were
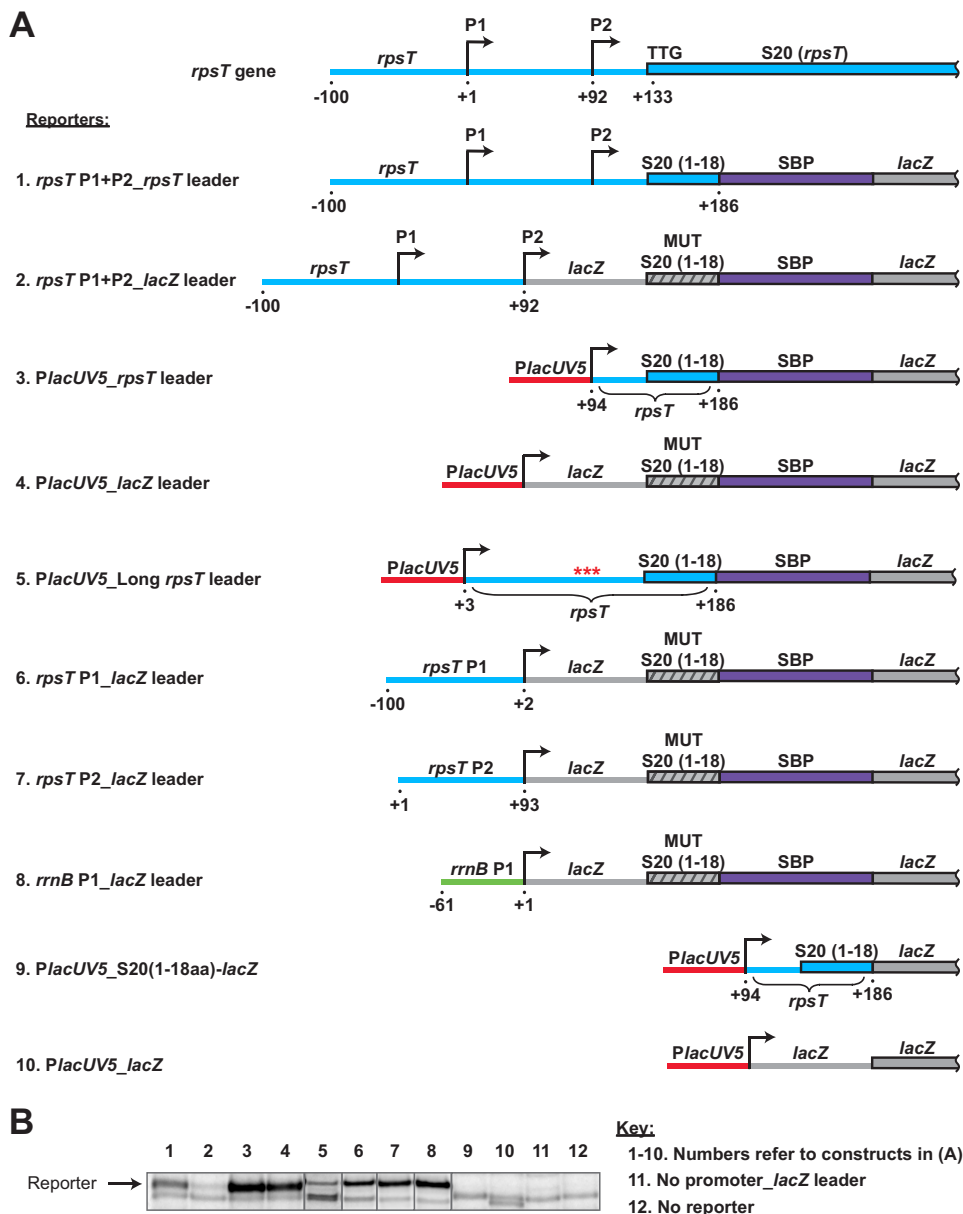
FIG 1 Reporters for measuring regulation of S20 r-protein synthesis. (A) A diagram of the regulatory region in the S20 r-protein operon is shown above schematics of the reporter constructs. Reporters 1 to 8 contain a fusion of the first 54 nucleotides (nt) of the *rpsT* ORF, coding for the first 18 amino acids (aa) of S20 (S20 [1–18]), in frame with an ORF encoding an SBP-*lacZ* fusion (S20 [1–18]-SBP-*lacZ*). We chose to fuse 54 nt (18 codons) of the *rpsT* ORF, instead of 18 nt (6 codons) like previous *rpsT* leader-*lacZ* fusions (25), to allow formation of the two hairpins that had been previously characterized in this region because they may have a role in translational feedback (26). The SBP (streptavidin-binding peptide)-*lacZ* fusion is described in the supplemental material. Constructs 1, 3, and 9 contain the wild-type *rpsT* leader sequence starting at the *rpsT* P2 transcription start site. Constructs 2, 4, and 6 to 8 contain a *trp-lacZ* leader instead of the *rpsT* leader, as well as an S20 (1–18)-SBP-*lacZ* fusion in which mutations were introduced into the S20 translation initiation region to eliminate regulation through translational feedback (MUT S20 [1–18]; details in the supplemental material). Constructs 6, 7, and 8 contain the *rpsT* P1, *rpsT* P2, and *rrnB* P1 promoters, respectively, fused to the *trp-lacZ* leader. Constructs 3 to 5, 9, and 10 contained the *lacUV5* promoter (48), a control promoter that is not regulated by ppGpp (7). Construct 5 contains the *lacUV5* promoter fused to the *rpsT* mRNA leader that corresponds to the leader transcribed from *rpsT* P1. Red asterisks represent 7 point mutations in the −35 and −10 elements of the *rpsT* P2 promoter designed to eliminate *rpsT* P2 activity (details in the supplemental material). The P*lacUV5* S20 (1–18)-*lacZ* and P*lacUV5_lacZ* reporters (constructs 9 and 10, respectively) contain the WT *lacZ* ORF and were used for size comparison to the S20 (1–18)-SBP-*lacZ* reporter protein. Numbers below the lines refer to positions relative to the *rpsT* P1 transcription start site. (B) Representative protein gels showing only the reporter protein products expressed from the constructs in panel A. A construct encoding SBP-*lacZ* but without a promoter was used to measure background levels of reporter protein expression (lane 11). The background strain used for reporter constructs, VH1000, is the "No reporter" control (lane 12).
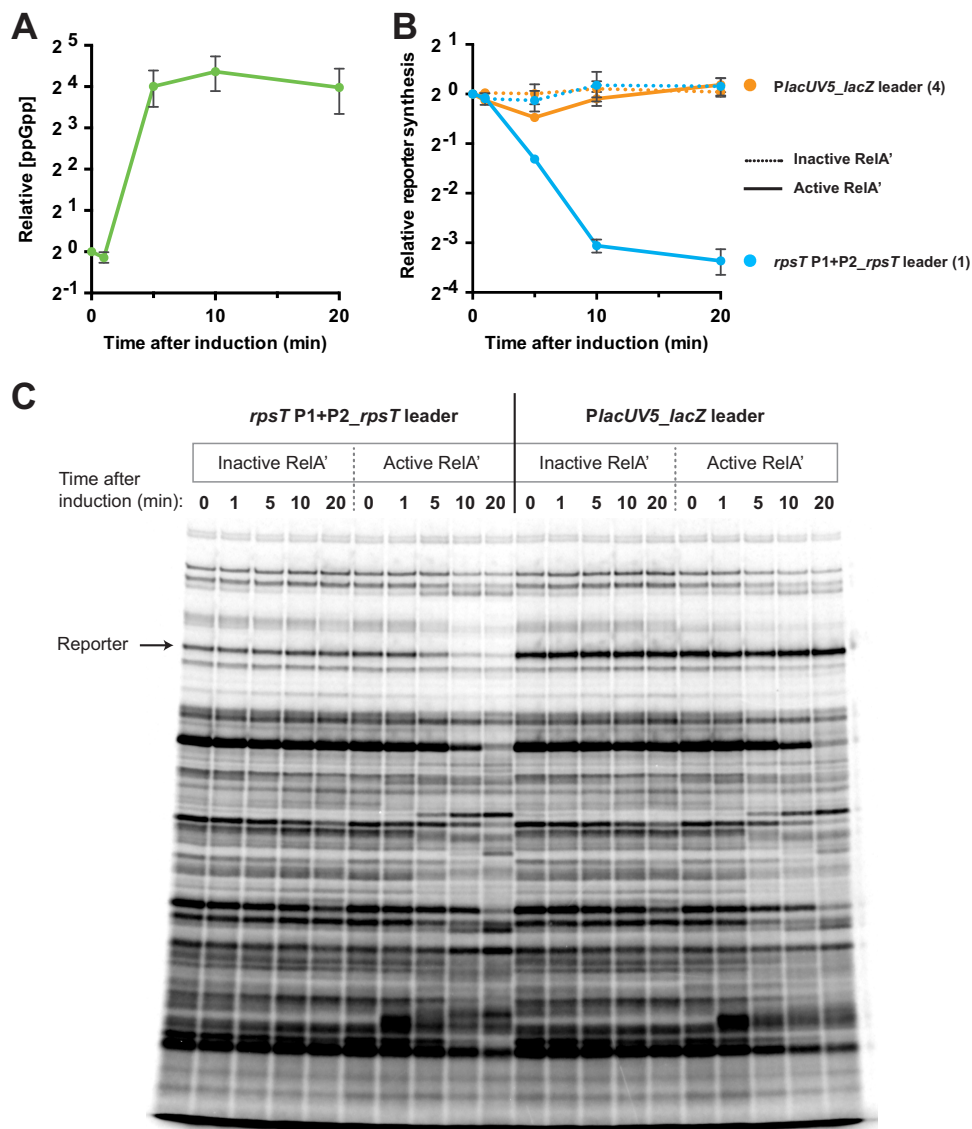
FIG 2 S20 synthesis during induction of ppGpp synthesis. (A) ppGpp levels were measured as described in Materials and Methods. Error bars represent the range ($n = 2$). Data are from chromatogram shown in Fig. 5B, lanes 1 to 5. Relative increases in ppGpp concentration (y axis) are likely an underestimate because the signal at time zero is close to background. (B) The reporter protein band from the strains containing the *rpsT* P1+P2_*rpsT* leader and P*lacUV5_lacZ* leader constructs (constructs 1 and 4 in Fig. 1A), and either the active or inactive RelA′ plasmid, was quantified by phosphorimaging, corrected for background, and normalized to the reporter band at time zero for each strain. Error bars represent the range ($n = 2$). Numbers in parenthesis in the labels refer to the numbers of the reporter constructs shown in Fig. 1A. (C) Representative gel illustrating ^{35}S-pulse-labeled protein profiles following ppGpp induction as described in the text and Materials and Methods. Arrow shows the position of the reporter band.

negligible. Our system made it possible to assay reporter synthesis in one step by phosphorimaging.

Changes in S20 r-protein synthesis following ppGpp induction. Previous work showed that synthesis of r-proteins, including S20, is regulated in a *relA*-dependent manner (29). To test the role of ppGpp in r-protein synthesis, we used an inducible plasmid that encodes a constitutively active version of the ppGpp synthetase RelA (RelA′) (30), whose expression resulted in rapid accumulation of ppGpp (Fig. 2A). Quantification of the reporter protein band during ppGpp induction indicated that there was an ~8-fold reduction in synthesis from the construct containing the *rpsT* promoters and the *rpsT* leader sequence (construct 1) at 10 min after induction,
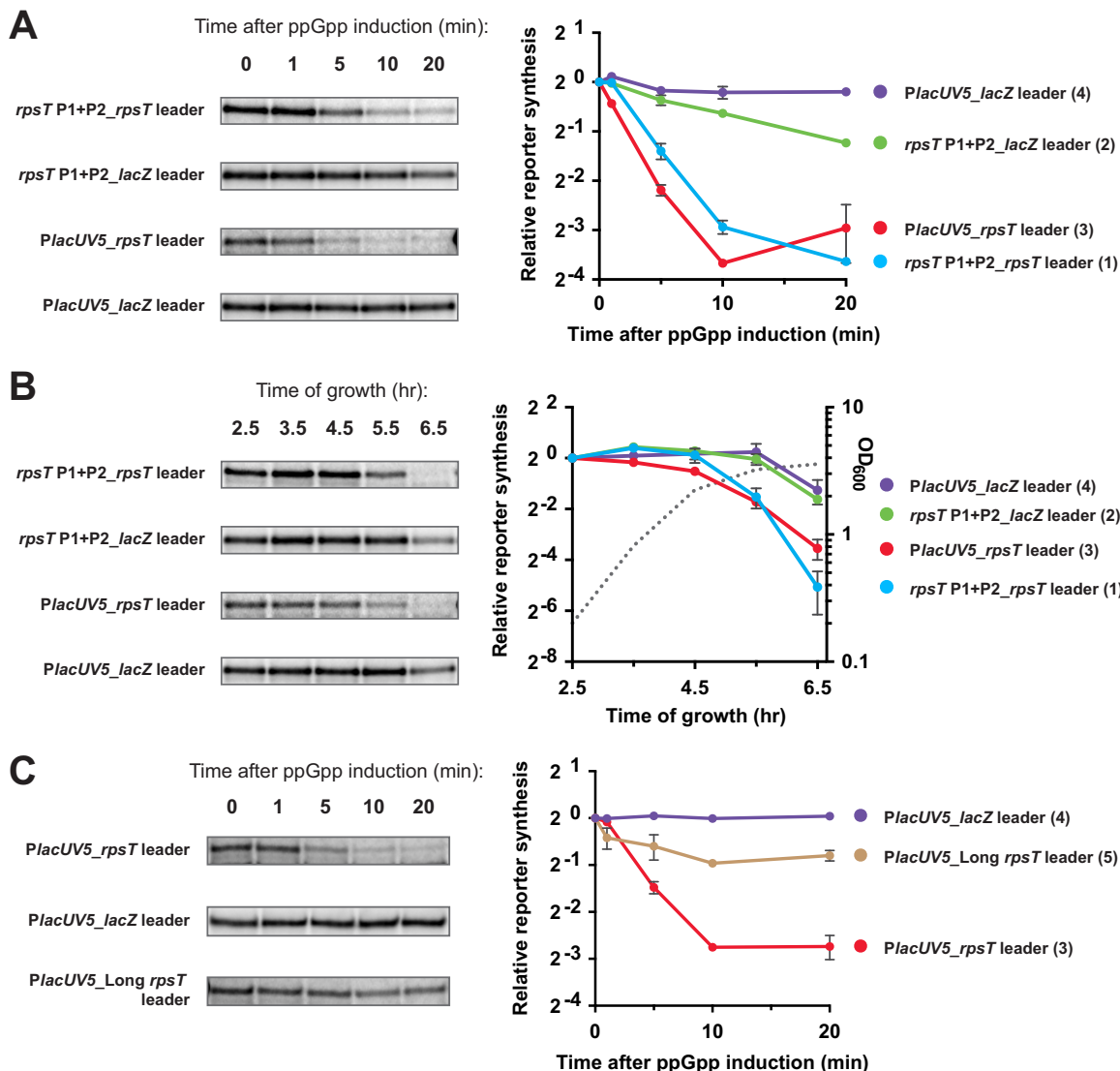
FIG 3 Regulation of S20 synthesis by ppGpp requires the *rpsT* leader. (A) ^{35}S reporter protein bands from a representative gel are shown from *rpsT* reporters following induction of ppGpp. Relative synthesis plots at the right are from multiple experiments. (B) Reporter protein bands and quantification from multiple experiments as in panel A during transition into stationary phase. Cells were grown in MOPS medium (44) supplemented with 19 aa (no methionine) and glucose as the carbon source. Pulse-labeled samples were taken every hour starting at an OD_{600} of 0.2 (see Materials and Methods). (C) Same as panel A but with the long *rpsT* leader reporter (Fig. 1A, construct 5). Error bars in each graph indicate the range (n = 2). Numbers in parenthesis in the labels refer to the numbers of the reporter constructs shown in Fig. 1A.

whereas ppGpp induction had no effect on the control construct containing the *lacUV5* promoter and *lacZ* leader (construct 4) (Fig. 2B and C). Expression of a catalytically inactive RelA′ had no effect on protein synthesis from either reporter strain (Fig. 2B and C).

Not surprisingly, ppGpp induction resulted in increases or decreases in synthesis of many proteins in the cell lysate (Fig. 2C), reflecting the global reprogramming of gene expression by ppGpp (reviewed in reference 31). These changes in the cellular protein profile served as an internal control for ppGpp induction. Taken together, our results show that our system allowed measurement of rapid sequence-dependent changes in reporter protein expression in response to changes in nutritional conditions.

ppGpp-dependent regulation of S20 synthesis primarily requires the *rpsT* leader. S20 reporter synthesis from reporters containing the *rpsT* leader (Fig. 3A, constructs 1 and 3) declined quickly in response to ppGpp induction (8-fold and 12-fold

at 10 min and 12-fold and 8-fold at 20 min, respectively) (blue and red curves). In contrast, ppGpp inhibited expression only slightly when the *rpsT* promoters were fused to the *lacZ* leader (construct 2, green curve) and not at all from the P*lacUV5_lacZ* leader construct (construct 4, purple curve). We also measured expression of the S20 reporter during entry into stationary phase (Fig. 3B), a transition when there is a temporary increase in ppGpp concentration and a steady decrease in the levels of NTPs (4). Expression from constructs with the *rpsT* leader declined more rapidly than from those with the *lacZ* leader during entry into stationary phase (Fig. 3B). Thus, ppGpp-dependent regulation of S20 synthesis occurs primarily at the translation level after shifts during exponential phase and during entry into stationary phase.

We based the upstream limit of the *rpsT* mRNA sequence in the P*lacUV5_rpsT* leader reporter (construct 3) on the *rpsT* P2 transcription start site because this promoter was the more active of the two *rpsT* promoters *in vivo* (32) and because the resulting mRNA contained the sequences necessary for regulation by translational feedback (25). However, the mRNA derived from the *rpsT* P1 promoter can in theory be regulated differently from that starting at P2, e.g., the sequences between *rpsT* P1 and P2 may potentially alter the secondary structure of the region responsible for translational feedback. To test regulation of the *rpsT* leader starting from *rpsT* P1, we constructed a reporter with the *lacUV5* promoter fused to the *rpsT* leader starting at +3 relative to *rpsT* P1 and with mutations that eliminated activity of the *rpsT* P2 promoter (P*lacUV5_Long rpsT* leader) (Fig. 1A, construct 5) (see the supplemental material for details).

ppGpp inhibited S20 synthesis about 2-fold from the reporter derived from the mRNA starting at *rpsT* P1, in contrast to the 8-fold inhibition observed with the reporter derived from the mRNA starting at *rpsT* P2 (Fig. 3C, constructs 5 and 3, respectively). We conclude that both mRNAs are regulated by translational feedback in response to ppGpp induction, but the *rpsT* P2-derived mRNA appears to be more sensitive to regulation than the *rpsT* P1-derived mRNA. In theory, the reduced translational feedback of the P1 transcript may derive from a difference in mRNA folding that interferes with S20 binding, from increased ribosome loading on the P2-derived transcript or from a shorter mRNA lifetime. Furthermore, we cannot exclude the possibility that the mutations introduced to inactivate *rpsT* P2 alter the structure of the mRNA and thus its regulation. In any case, the increased sensitivity of the two transcripts to ppGpp was not a property of the promoter.

The *rpsT* promoters are regulated *in vivo* but to a lesser extent than rRNA promoters. The results shown in Fig. 3A and B indicated that ppGpp regulates S20 expression primarily at the translational level rather than by regulating *rpsT* promoter activity. However, using different methods for measuring promoter activity and for inducing ppGpp accumulation than those used here (19), we showed previously that the individual *rpsT* P1 and P2 promoters were regulated directly by ppGpp (see Discussion). Using the same reporter system and ppGpp induction conditions as in Fig. 3A, we found that there was no effect of ppGpp on the *rpsT* P1 promoter (Fig. 4A, construct 6) and a 2-fold effect on *rpsT* P2 (Fig. 4A, construct 7). This contrasts with the 8- to 12-fold effect of ppGpp on expression when the *rpsT* leader was present (Fig. 3A, constructs 1 and 3) and the 16-fold effect of ppGpp on the *rrnB* P1 promoter (Fig. 4A, construct 8). Thus, under conditions in which effects of ppGpp on the *rpsT* promoters and the translational feedback mechanism can be compared directly, effects at the translation level dominated the *rpsT* response to ppGpp. The magnitude of ppGpp-dependent inhibition from constructs that contain the *rpsT* leader is similar to that observed with the *rrnB* P1 promoter (compare Fig. 3A, constructs 1 and 3, to Fig. 4A, construct 8), supporting a model in which direct regulation of rRNA transcription results in indirect regulation of r-protein synthesis through translational feedback (see Discussion).

We next tested if regulation of the *rpsT* promoters may play a larger role in regulation of S20 synthesis during outgrowth from stationary phase, a condition when rRNA promoter activities are dependent almost exclusively on a surge in intracellular
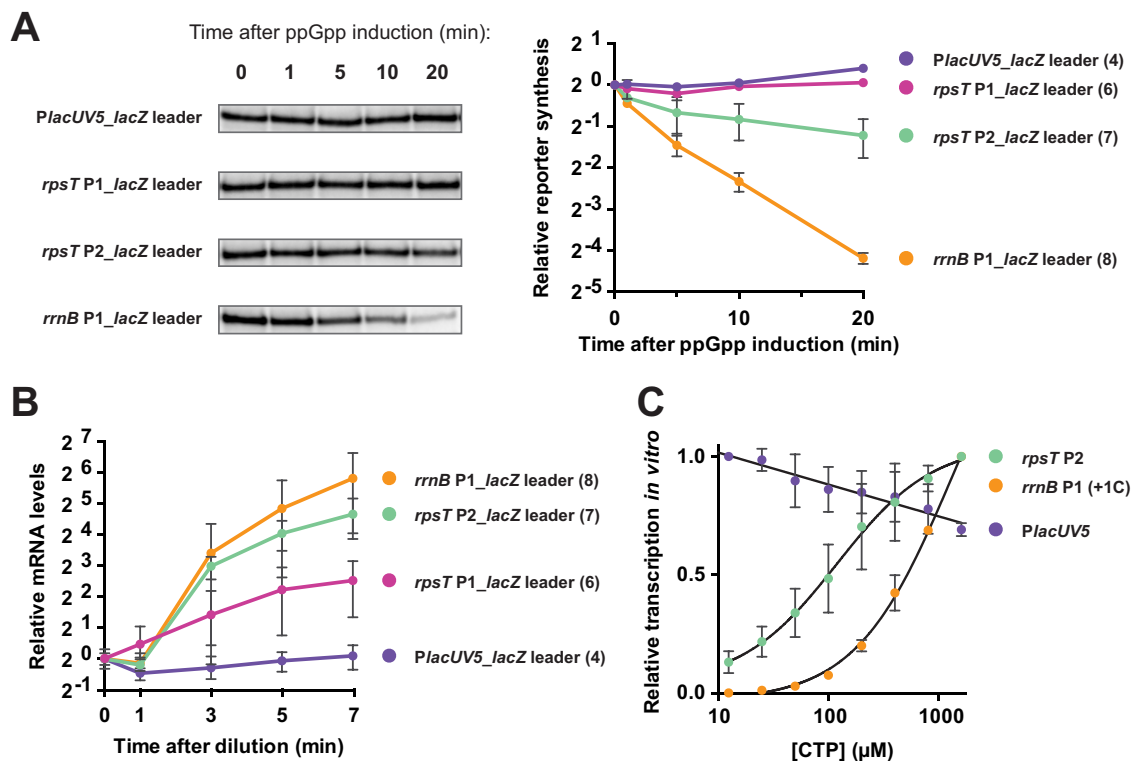
FIG 4 Regulation of *rpsT* promoters. (A) ³⁵S reporter protein bands from representative gel and quantification from multiple experiments showing changes in reporter synthesis from transcriptional fusions (Fig. 1A, constructs 4 and 6 to 8) following induction of ppGpp *in vivo*. Promoter endpoints of the transcriptional fusions are P*lacUV5* (−59/+1), *rpsT* P1 (−100/+2), *rpsT* P2 (−90/+2), and *rrnB* P1 (−61/+1), where +1 is the transcription start site. Error bars represent the range (n = 2). (B) Promoter activity during outgrowth was measured by RT-qPCR and was initiated by a 1:10 dilution of a stationary-phase culture into fresh medium. Error bars represent the range (n = 2). (C) Promoter activity measured by *in vitro* transcription at different CTP concentrations. Promoter fragments with the indicated endpoints relative to the transcription start site (*rpsT* P2, −89/+50; *rrnB* P1 + 1C, −66/+9; and P*lacUV5*, −59/+38) were cloned into pRLG770, resulting in plasmids pRLG9237, pRLG3735, and pRLG2222, respectively (for details, see Table S1 in the supplemental material). Error bars represent the SD (n = 3). Numbers in parenthesis in the labels refer to the numbers of the reporter constructs shown in Fig. 1A.

NTP pools rather than on decreases in the already very low concentration of ppGpp (4). Using reverse transcriptase quantitative PCR (RT-qPCR) to measure mRNA levels from the reporter constructs used in Fig. 4A, we found that the activities of the *rpsT* P1 and P2 promoters, like that of the *rrnB* P1 promoter, increased rapidly after dilution of overnight cultures into fresh medium (Fig. 4B). *rrnB* P1 increased the most, *rpsT* P2 was next, then *rpsT* P1, and P*lacUV5* increased little if at all, following the same rank order of the responses of these constructs to ppGpp induction (Fig. 4A).

Since sensitivity to the concentration of its initiating nucleotide is responsible for the increase in *rrnB* P1 activity during outgrowth (4), we tested the dependence of *rpsT* P2 activity on initiating NTP (iNTP) concentration *in vitro*. *rpsT* P2 starts predominantly with CTP (32); therefore, we used an *rrnB* P1 mutant that starts with CTP (instead of ATP) for comparison. *rpsT* P2 activity depended on CTP concentration, unlike the control promoter *lacUV5*, but its dependence was not as great as that for *rrnB* P1 (+1C) (Fig. 4C). Since the concentration of CTP increases during outgrowth like the other NTPs (4, 33), we suggest that the dramatic increase in expression of *rpsT* P2 during outgrowth results, at least in part, from the increase in iNTP concentration.

Control of S20 synthesis requires regulation of rRNA transcription. The most straightforward explanation for the observed *rpsT* leader-dependent regulation of S20 synthesis by ppGpp is that S20 regulates its own synthesis at the level of translation in response to changes in free rRNA levels that titrate away S20 during ribosome assembly (22). In this model, the effect of ppGpp on S20 synthesis is indirect, a consequence of the direct regulation of rRNA synthesis by ppGpp. Nevertheless, it remained a formal
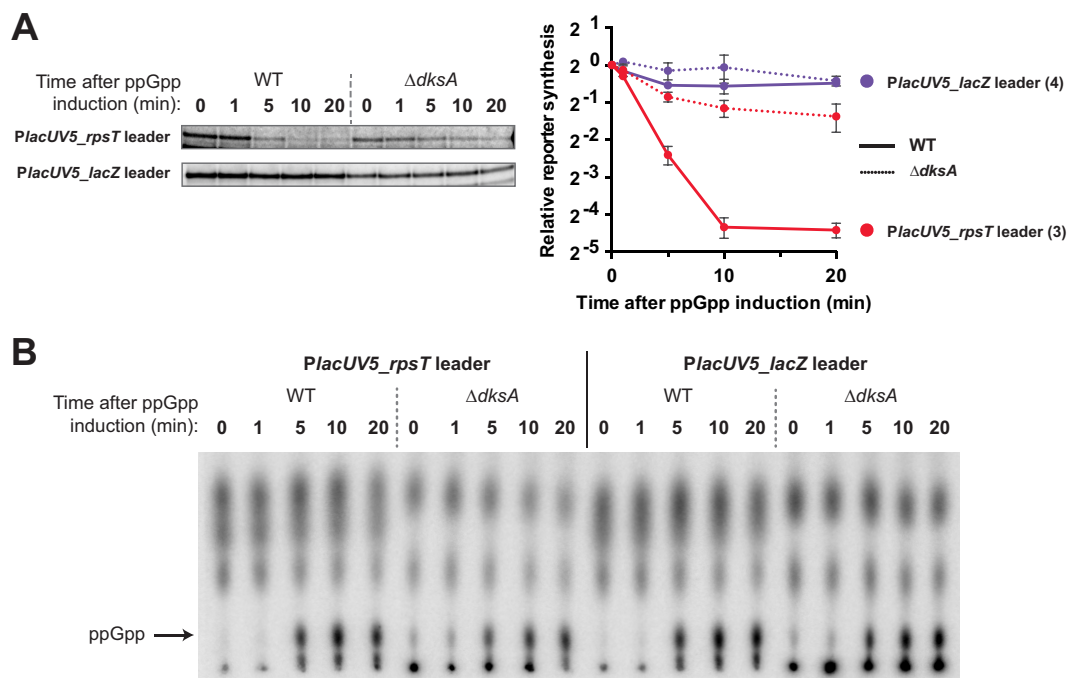
FIG 5 DksA is required for ppGpp-dependent regulation of S20 synthesis. (A) To the left is a representative gel showing 35S reporter bands from the P*lacUV5_rpsT* leader and P*lacUV5_lacZ* leader constructs at times after ppGpp induction in either a WT or Δ*dksA* strain background. To the right is the quantification of reporter protein synthesis from multiple experiments (*n* = 2). Numbers in parenthesis in the labels refer to the numbers of the reporter constructs shown in Fig. 1A. Error bars represent the range (*n* = 2). (B) Thin-layer chromatogram shows measurement of ppGpp levels following induction of *relA'* expression from pALS13 in the strains used in panel A (*n* = 2). ppGpp indicates the region of the plate where ppGpp and pppGpp run together.

possibility that ppGpp regulated S20 synthesis by some other mechanism that did not require regulation of rRNA transcription.

Therefore, we tested the effect of ppGpp on S20 synthesis in a strain in which rRNA was derepressed because it lacked DksA (Δ*dksA*) (10, 11). In agreement with the results shown in Fig. 3 and 4, synthesis of the reporter from the construct containing the *rpsT* leader was strongly decreased by 20 min after induction of ppGpp synthesis in a wild-type (WT) strain (Fig. 5A, solid red curve), but there was only a small decrease in the strain lacking *dksA* (Fig. 5A, red dotted curve). As expected, ppGpp induction had no effect on the P*lacUV5_lacZ* leader construct in the wild-type or Δ*dksA* strain. Not surprisingly, DksA affected the synthesis of many other cellular proteins (see Fig. S1 in the supplemental material), consistent with the role of ppGpp/DksA in global reprogramming of gene expression (11).

There was a formal possibility that the lack of strong effects of ppGpp induction in the Δ*dksA* strain resulted from a failure to induce ppGpp synthesis, not from a defect in regulation of rRNA synthesis. Therefore, we measured ppGpp induction directly by 32P-orthophosphate incorporation and thin-layer chromatography. ppGpp levels were similar or identical to those in the wild-type strain (Fig. 5B). Taken together, the data suggest that effects of ppGpp on reporters containing the *rpsT* leader occurred indirectly, likely through translational feedback inhibition of S20 synthesis, in response to inhibition of rRNA synthesis by ppGpp/DksA.

The *rpsJ* promoter is dispensable for ppGpp-dependent regulation of S10 synthesis. Previously, we reported that the *rpsJ* (S10) promoter was one of the r-protein promoters most strongly affected by DksA and ppGpp *in vitro* and by the absence of DksA in stationary phase (19). The S10 operon is regulated by the r-protein L4, which binds to the leader region of its mRNA (34), causing inhibition of translation initiation and premature transcription termination (reviewed in reference 3). We used the same strategy as with *rpsT* but using reporters based on *rpsJ* (Fig. 6A) to examine the relative
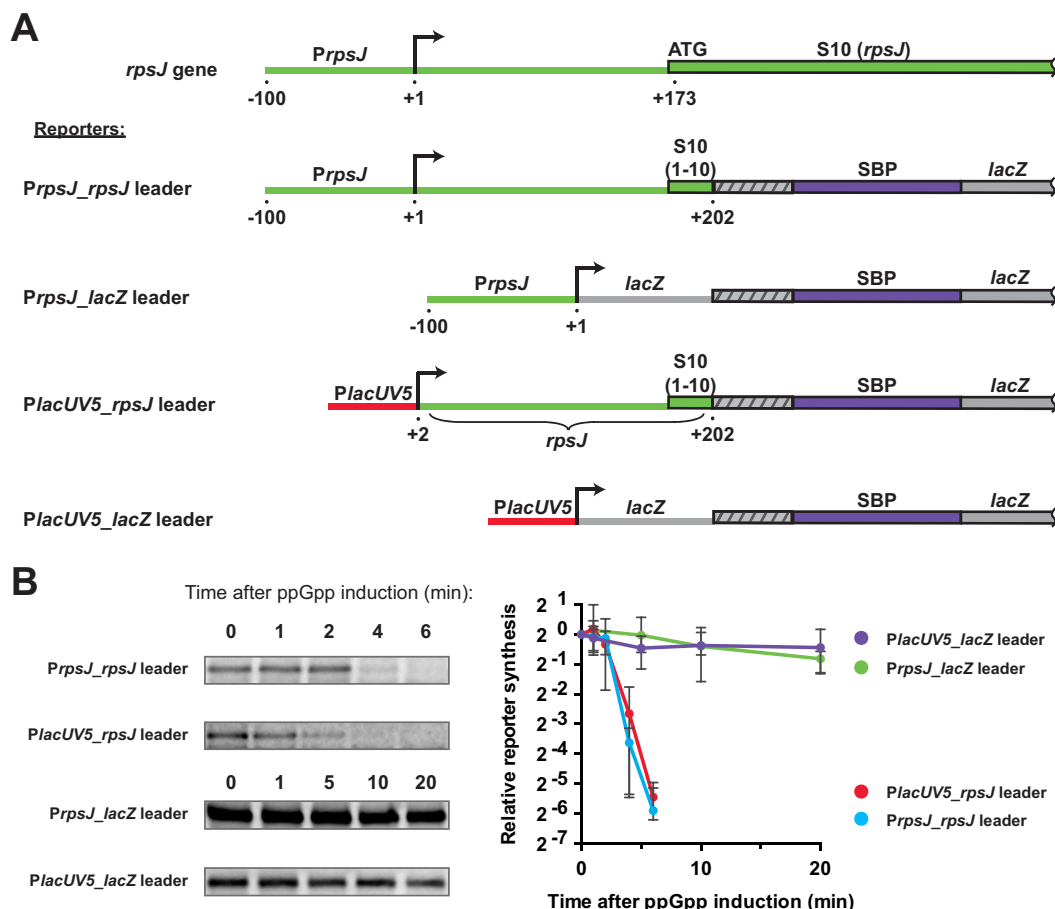
FIG 6 Regulation of the S10 operon is independent of the *rpsJ* promoter. (A) Schematic diagrams of the 5′ region of the S10 operon and the *rpsJ* reporters. Numbering below diagrams is relative to the P*rpsJ* transcription start site (49). Reporters containing the *rpsJ* leader are N-terminal fusions of the *rpsJ* leader and the first 30 nt of the *rpsJ* ORF, which code for the first 10 aa of S10 (S10 [1–10]) to the MUT S20 (1–18)-SBP-*lacZ* reporter (described in the supplemental material). The *lacUV5_lacZ* leader construct is the same one shown in Fig. 1A. (B) ^{35}S reporter protein bands from representative gel and quantification from multiple experiments following induction of ppGpp. Error bars represent the standard deviation ($n = 3$).

contribution of the *rpsJ* promoter versus the leader region to regulation by ppGpp. Reporter synthesis was even more strongly and rapidly inhibited in response to ppGpp induction when the reporter contained the *rpsJ* leader than when the reporter contained the *rpsT* leader (Fig. 6B; compare with Fig. 3A), whether or not the reporters contained the *rpsJ* promoter (Fig. 6B). We conclude that the S10 promoter complex can be directly inhibited by ppGpp (19), but the initial and predominant effect of ppGpp during nutritional shifts is indirect, stemming from effects of ppGpp on rRNA synthesis and L4-mediated inhibition through attenuation and translational feedback.

DISCUSSION

Regulation of ribosomal protein synthesis in *E. coli* occurs through a translational feedback mechanism that matches r-protein output to rRNA synthesis rates (reviewed in references 1, 3, and 35). Nevertheless, there is considerable evidence that transcription of many r-protein operons is regulated directly by DksA and ppGpp (19). Here, we attempted to determine whether these systems are redundant, with each one capable of providing full regulatory function when the other is inactivated, or whether the two systems serve different functions, with both mechanisms retained in evolution because they regulate r-protein synthesis at different times in growth or under different nutritional conditions. We studied regulation of the S20 and S10 operons as test cases because it was shown previously that they were regulated by both transcriptional and

translational mechanisms and because the sequences required for each type of regulatory mechanism were known (3, 19).

Even though the *rpsT* and *rpsJ* promoters were among the r-protein promoters most strongly inhibited by ppGpp and DksA in our previous study (19), we found that the sequences responsible for translational feedback were actually the ones most required for regulation of r-protein synthesis; these sequences were both necessary and sufficient for rapid changes in r-protein synthesis in response to ppGpp. Nevertheless, the r-protein promoters were regulated but to a smaller degree than the rRNA promoters. Following production of ppGpp by induction of RelA' synthesis from a plasmid, *rrnB* P1 was 7.2-fold more inhibited by ppGpp than *rpsT* P2 *in vivo* (Fig. 4A), consistent with the different sensitivities of r-protein and rRNA promoters to regulation by DksA *in vitro* that we reported previously (50% inhibitory concentration [IC_{50}] for DksA in inhibiting transcription from *rrnB* P1 was ∼5-fold lower than the IC_{50} for *rpsT* P2) (19). Thus, the emerging model for responses to rapid shifts in nutrient conditions in exponentially growing cells is that r-protein promoters respond to ppGpp *in vivo*, but this response is fairly small compared to the responses of rRNA promoters to ppGpp, which in turn lead to regulation of r-protein synthesis through translational feedback (19).

When considered in the context of the much greater extent of inhibition when the *rpsT* leader is present (and therefore subject to translational control), the effects of ppGpp/DksA on the *rpsT* promoters are small. However, they are not insignificant. The extent of regulation of the promoters was quite similar in reference 19 to that reported here. There was ∼3-fold inhibition of *rpsT* P2 upon ppGpp induction in Fig. 2E in reference 19 versus ∼2-fold inhibition here in Fig. 4A. For *rpsT* P1, there was little or no effect of ppGpp induction on *rpsT* P1 here (Fig. 4A) and an ∼3-fold effect in Fig. 2D of reference 19. These differences are attributable, at least in part, to differences in the levels of ppGpp in the two studies: ppGpp levels are about 1.5-fold greater after amino acid starvation as performed in reference 19 (∼900 pmol/A_{600} [36] compared to ∼600 pmol/A_{600} from utilizing an inducible *relA'* plasmid like that used here [37]).

Likewise, regulation of the *rpsJ* operon during log phase was dependent on transcript leader sequences rather than on the promoter (Fig. 6B), consistent with the absence of an effect of DksA on regulation of the *rpsJ* promoter during log phase (Fig. 1B in reference 19). As noted previously (19), the *rpsJ* promoter was regulated by ppGpp/DksA in stationary phase and *in vitro*, phenomena that will require further study.

There are times during growth when r-protein promoters, including those for the *rpsT* and *rpsJ* operons, play an important role in regulation of r-protein synthesis, namely, during stationary phase and outgrowth from stationary phase (19). We found that *rpsT* P2 promoter activity rapidly increased during outgrowth and was dependent on high levels of the initiating NTP *in vitro* (Fig. 4B and C) as reported previously for rRNA promoters (4). We suggest that the rapid increase in NTP levels that occurs during outgrowth is responsible for turning on r-protein synthesis, which together with increased rRNA synthesis results in rapid synthesis of ribosomes when nutritional conditions improve.

Two different mechanisms regulate *E. coli* rRNA promoter activity during growth. rRNA promoters are regulated by ppGpp during nutritional shifts in log phase and by NTP concentrations during stationary phase and outgrowth from stationary phase (4). The rationale for having two different mechanisms is that NTP concentrations are saturating in log phase; they are too high and buffered against fluctuations and thus cannot account for the rapid responses in rRNA promoter activity that occur during nutritional shifts in log-phase cells (38). Conversely, ppGpp is a negative regulator of rRNA promoters, but the concentration of ppGpp is too low in stationary phase to effectively regulate transcription of rRNA. Thus, a further decrease in ppGpp concentration cannot account for the rapid increase in promoter activity during outgrowth (4). We propose that having two mechanisms for regulation of r-protein synthesis has a similar basis as that for rRNA promoters: ppGpp is responsible for rapid changes in r-protein synthesis following nutritional shifts in log phase, and NTP concentrations regulate r-protein expression during stationary phase and outgrowth.

The primary difference between regulation of rRNA and r-protein synthesis is that the effect of ppGpp on r-protein synthesis during nutritional shifts in log phase is indirect, occurring primarily through translational feedback in response to changes in rRNA synthesis. However, translational feedback would be impractical for maintaining inhibition of r-protein synthesis during metabolic dormancy because there is a lack of robust translation during stationary phase (39) and most free r-proteins are unstable when not incorporated into ribosomes (40), which together would result in the concentration of repressor r-proteins being too low to inhibit r-protein synthesis. Thus, we propose that low NTP levels are responsible for maintaining inhibition of r-protein expression during stationary phase, with NTP levels increasing rapidly to turn on ribosome synthesis when nutritional conditions improve, as with rRNA promoters (4).

We have attributed the requirement for the *rpsT* (S20) leader in regulation by ppGpp to its role as the target site for translational feedback inhibition by S20 (3, 25), a model in which the requirement for DksA (Fig. 5A) is explained by its role in regulation of rRNA transcription initiation. It was reported previously that overexpression of S20 from a plasmid reduced expression from an *rpsT* leader/RBS-*lacZ* fusion and that S20 inhibited its own synthesis in a coupled transcription-translation system (23, 24). Mutations in the *rpsT* leader and start codon affected translational feedback (25), suggesting that this inhibition occurred at the translation level (15) and is consistent with the simple model that S20 regulates translation from its own mRNA. However, we note that there is currently no evidence for direct binding of S20 by itself to its mRNA (21). Alternative models consistent with our data are that S20 may bind to the *rpsT* mRNA as a complex with other proteins, that S20 may target the translation initiation complex rather than compete with ribosome binding, or that feedback inhibition may result from S20-dependent transcription attenuation. Discriminating among these possible mechanisms will require further investigation.

MATERIALS AND METHODS

Strain constructions. See the supplemental material for more details about strain construction. Strains, plasmids, oligonucleotides, and synthetic gene fragments (gBlocks) used in this study are listed in Tables S1 and S2 in the supplemental material. Synthetic DNAs were obtained from Integrated DNA Technologies (IDT). Reporter fusions were constructed in strain DY330 by recombineering and then transferred into VH1000 by P1 transduction (41, 42). The *dksA* gene was deleted by infection of reporter strains with a P1 lysate grown on RLG6632 (*dksA::kan*) and selection for kanamycin resistance. The strains were transformed with plasmids as described previously (43).

Reporter protein synthesis. Cells were grown at 37°C with aeration in morpholinepropanesulfonic acid (MOPS) medium supplemented with 0.4% glucose, 80 μg/ml 19 amino acids (no methionine), and 10 μg/ml thiamine (44). Experiments with $\Delta dksA$ strains were performed with the same medium as above, but amino acids were added from 5× Supplement EZ minus methionine (Teknova; M2109). For measuring regulation of reporter protein synthesis during ppGpp induction, cells carrying either the pALS13 or pALS14 plasmids (30), maintained with 100 μg/ml ampicillin, were grown to mid-log phase (optical density at 600 nm [OD_{600}], ~0.2), induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and samples for pulse-labeling were taken immediately before and at the indicated times after addition of IPTG. Cells were pulse-labeled by incubating 0.5-ml aliquots of the cultures with 10 μCi of L-[^{35}S]methionine (PerkinElmer; NEG709A) for 10 min at 37°C. Under these conditions, cells saturated for incorporation of radioactivity into protein within 30 s of L-[^{35}S]methionine addition (H. L. Burgos and R. L. Gourse, unpublished data). Cells were pelleted by centrifugation, suspended in sample buffer containing SDS (28), and lysed by heating to 65°C for 10 min. The cell lysate was then vortexed vigorously to shear genomic DNA and cleared by centrifugation. The cleared lysate was run in an 8% acrylamide discontinuous buffer SDS-PAGE system (28) and visualized by phosphorimaging.

For measuring reporter protein synthesis during entry into stationary phase, cultures were inoculated at an OD_{600} of ~0.01 and grown in the same medium as described above. A sample for pulse-labeling was taken when the cells reached an OD_{600} of ~0.2 and every hour after. The volume of culture harvested at each time point was adjusted to the same OD_{600} to ensure that labeling was performed with the same number of cells. Radiolabeled proteins were visualized and measured as described above.

Analysis of mRNA levels during outgrowth. Cells were grown at 37°C with aeration in MOPS defined medium supplemented with 0.4% glucose, 80 μg/ml of all 20 amino acids, and 10 μg/ml thiamine (44). To measure regulation of promoter activity during outgrowth, we inoculated cultures at an OD_{600} of ~0.001, allowed them to grow for 24 h, then initiated outgrowth by diluting the stationary-phase culture 1:10 into fresh medium prewarmed to 37°C. RNA extraction and RT-qPCR were performed as described previously (10). Briefly, samples for RNA extraction were removed from the stationary-phase culture and at the indicated times after dilution into fresh medium by transferring aliquots into an ice-cold 95% ethanol–5% phenol stop solution that inactivates RNases (45). Cells were lysed in Tris-EDTA

(TE) buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA) containing lysozyme (Epicentre), 1% SDS, and total RNA from strain RLG11387 (contains plasmid P*lacUV5*_GFP) as a marker for RNA loss during extraction. RNA was extracted from the cells using the hot phenol method (45) and digested with DNase I (NEB). cDNA was synthesized from 0.5 μg of total RNA using the iScript cDNA synthesis kit (Bio-Rad; 170-8891). Quantitative PCR was performed with the cDNA using the i*Taq* Universal SYBR green supermix (Bio-Rad; 172-5122) on a CFX Connect real-time PCR detection system (Bio-Rad). The mRNA originating from the reporter fusion was detected using oligonucleotides 7664 and 7665, and the *gfp* mRNA was detected with oligonucleotides 7608 and 7609 (Table S2). The amount of reporter mRNA was normalized to the *gfp* mRNA and to the preshift samples using the $2^{-\Delta\Delta C_T}$ method (46).

In vitro transcription. Multiple-round *in vitro* transcription reactions were performed essentially as described previously (47). Reactions were carried out at 30°C and contained 170 mM KCl, 10 nM Eσ^{70} RNAP, 1 nM supercoiled plasmid template, 200 μM ATP and GTP, 10 μM UTP, and ~2 μCi of [γ-^{32}P]UTP. CTP was added to the reactions in various concentrations ranging from 12.5 μM to 1.6 mM. The promoters of interest were contained in the pRLG770 transcription vector (plasmids are listed in Table S1). Transcripts were resolved by electrophoresis in a 5.5% polyacrylamide, 7 M urea gel and visualized by phosphorimaging.

Measurement of ppGpp levels. Cells were grown in the same medium as described above in "Reporter protein synthesis" for $\Delta dksA$ strains, supplemented with 100 μg/ml ampicillin to maintain the pALS13 plasmid and 10 μCi/ml of [^{32}P]orthophosphate. At an OD$_{600}$ of ~0.2, cells were induced with 1 mM IPTG, and samples were harvested before and at the indicated times after induction. Samples were processed by extraction with formic acid and analyzed by thin-layer chromatography in 0.85 M KH$_2$PO$_4$ (pH 3.4) buffer as described previously (10).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB .00407-17.

SUPPLEMENTAL FILE 1, PDF file, 1.0 MB.

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