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Active Transcription of rRNA Operons Is a Driving Force for the Distribution of RNA Polymerase in Bacteria: Effect of Extrachromosomal Copies of *rrnB* on the In Vivo Localization of RNA Polymerase

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In contrast to eukaryotes, bacteria such as *Escherichia coli* contain only one form of RNA polymerase (RNAP), which is responsible for all cellular transcription. Using an RNAP-green fluorescent protein fusion protein, we showed previously that *E. coli* RNAP is partitioned exclusively in the nucleoid and that stable RNA synthesis, particularly rRNA transcription, is critical for concentrating a significant fraction of RNAP in transcription foci during exponential growth. The extent of focus formation varies under different physiological conditions, supporting the proposition that RNAP redistribution is an important element for global gene regulation. Here we show that extra, plasmid-borne copies of an rRNA operon recruit RNAP from the nucleoid into the cytoplasmic space and that this is accompanied by a reduction in the growth rate. Transcription of an intact rRNA operon is not necessary, although a minimal transcript length is required for this phenotype. Replacement of the ribosomal promoters with another strong promoter, P*tac*, abolished the effect. These results demonstrate that active synthesis from rRNA promoters is a major driving force for the distribution of RNAP in bacteria. The implications of our results for the regulation of rRNA synthesis and cell growth are discussed.

Escherichia coli RNA polymerase (RNAP) is a multisubunit enzyme consisting of $\alpha_2\beta\beta'\sigma$. Although there are seven σ factors in *E. coli*, σ^{70} predominates and is responsible for transcription of most genes (5). Unlike eukaryotes, in which three different RNAPs (PolI, PolII, and PolIII) (23) synthesize three different RNA species (rRNA, mRNA, and tRNA/5S rRNA, respectively), the single *E. coli* RNAP synthesizes all RNA and does so differentially in response to growth conditions (4, 21). For example, under optimal growth conditions, the vast majority of RNAP molecules synthesize rRNA and tRNA (termed stable RNAs), although the corresponding genes represent less than 1% of the genome. The remaining RNAP molecules are responsible for synthesizing the appropriate mRNAs from the other ~4,300 genes. Under suboptimal conditions, such as growth in nutrient-poor media, a far smaller fraction of the RNAP molecules is needed to synthesize sufficient amounts of stable RNAs, which allows expansion of new gene transcription. When cells are shifted from nutrient-rich conditions to starvation conditions, such as amino acid starvation, there is a stringent response, which results in dramatic reprogramming of transcription such that the expression of stable RNAs is inhibited while amino acid biosynthetic operons are activated (7). The redistribution of RNAP according to growth conditions has been proposed to be a key feature of global gene regulation during processes such as the stringent (nutrient starvation) response and carbon source limitation responses (1, 16, 29).

Recently, we studied the location and distribution of RNAP under different physiologic conditions by using a functional *rpoC-gfp* gene fusion on the *E. coli* chromosome and imaging RNAP-green fluorescent protein (GFP) in vivo by fluorescence microscopy (6). Indeed, while RNAP is located exclusively either within and/or surrounding the nucleoid (i.e., there is no RNAP-GFP signal in the cytoplasmic space), RNAP distribution is dynamic and sensitive to growth conditions. In particular, RNAP in fast-growing cells forms several transcription foci within the nucleoid. We proposed that these foci are transcription "factories" that actively synthesize stable RNAs, forming a structure(s) analogous to the eukaryotic nucleolus (8).

The study mentioned above indicated that synthesis of stable RNAs, particularly rRNA synthesis, is a driving force for RNAP distribution inside the cell. In this study, we determined the effect of extrachromosomal copies of an rRNA operon on the location and distribution of RNAP. We chose two plasmids for our initial study: pNO1301, which contains an intact *rrnB* operon, and pNO1302, which harbors an *rrnB* operon with a partial deletion. The pNO1301 and pNO1302 plasmids were used previously to study the effects of rRNA operon copy number on rRNA synthesis (14, 27). These plasmids are derivatives of pBR322, which has been reported to localize in the cytoplasm of *E. coli* cells (9, 20, 22). Our results demonstrate that active rRNA synthesis plays a pivotal role in the in vivo distribution of RNAP. In addition, our results suggest an ad-

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TABLE 1. Plasmids used

Plasmid	Description	Reference or source
pBR322	Cloning vector	3
pKK223-3	Expression vector	Pharmacia
pNO1301	Includes *rrnB* whole operon	14
pNO1302	pNO1301 derivative with deletion of a SalI-SalI fragment in the *rrn* operon	14
pDJ2754-11	pNO1301 derivative lacking *rrnB* P1 and *rrnB* P2	This study
pJ2754-17	pNO1301 derivative lacking *rrnB* P2	This study
pDJ2754-15	pNO1301 derivative lacking *rrnB* P1	This study
pDJ2790	pKK223-1 derivative lacking the *tac* promoter	This study
pDJ2791-A	Fragment of the *rrnB* operon including *rrnB* P1 and *rrnB* P2 plus a 1,800-nucleotide transcript cloned into pDJ2790	This study
pDJ2791-B	Fragment of the *rrnB* operon including *rrnB* P1 and *rrnB* P2 plus a 1,000-nucleotide transcript cloned into pDJ2790	This study
pDJ2845	Promoterless fragment of the *rrnB* operon (encoding the whole 16S rRNA) cloned into the pKK223-1 vector	This study

ditional cause for the inhibitory effect of extra rRNA gene copies in *trans* on chromosomal rRNA synthesis.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Strain DJ2735 is a DJ2599 (MG1655 *rpoC-gfp* Amp^r) derivative in which the Amp^r allele was replaced by the *cat* (Cm^r) gene using a high-efficiency λ Red recombination system (28). Briefly, a PCR fragment containing the Cm^r gene was synthesized from plasmid pACYC184 with oligonucleotides JC233A (5′ ATG AGT ATT CAA CAT TTC CGT GTC GCC CTT ATT CCC TTT TTT GCG GCA TTT ACC TGT GAC GGA AGA TCA CTT CGC 3′) and JC233B (5′ TTA CCA ATG CTT AAT CAG TGA GGC ACC TAT CTC AGC GAT CTG TCT ATT TCT TAA GGG CAC CAA TAA CTG CC 3′) (nucleotides identical to nucleotides in the Amp^r gene are underlined). This PCR fragment was recombined into the chromosome of cells carrying the *rpoC-gfp* Amp^r allele and a copy of the λ Red recombination system. Like DJ2599, strain DJ2735 is temperature sensitive for growth at temperatures above 37°C, likely due to a problem with GFP folding at high temperatures (11, 15, 24). The doubling time of DJ2735 in Luria-Bertani (LB) medium at 30°C is approximately 45 min.

The basic bacterial techniques used have been described elsewhere (18). All cultures were grown with vigorous agitation in a water bath at 30°C. Fresh overnight cultures were diluted 1/500 into fresh media. Cells were grown in M63 medium supplemented with glucose (final concentration, 0.2%) or in LB medium. Samples used for microscope observation were removed at an optical density at 600 nm of approximately 0.4.

Plasmid construction. The plasmids used in this work are listed in Table 1. All recombinant DNA techniques used were based on protocols described by Maniatis et al. (17). All PCRs were carried out using plasmid pNO1301 as a template.

To construct plasmids carrying promoterless or one-promoter copies of the *rrnB* gene, a 1.6-kbp NaeI-BglII fragment that carried both the *rrnB* P1 and P2 promoters in pNO1301 was replaced with DNA fragments synthesized by PCR and digested with NgoMIV and BglII as described below. Plasmid pDJ2754-17 containing a PCR fragment with the *rrnB* P1 promoter was obtained in two steps. First, two independent DNA fragments (fragments A and B) which share a complementary sequence (indicated by boldface type) at their 3′ and 5′ ends were each amplified by PCR. The primers used to amplify fragment A were JC254A (5′ ATT CTG ACT GTA GCC GGC GAT TAA ACC TGC TGC ACG 3′) for the upper strand and JC254E (5′ **TGC CGT TGT TCC GTG TCA GT**G GTG GCG CAT TAT AGG GAG 3′) for the lower strand. The primers used to amplify fragment B were JC254F (5′ **ACT GAC ACG GAA CAA CGG CA**A AAT TGA AGA GTT TGA TCA TGG C 3′) for the upper strand and JC254B (5′ GGT ATT CCT CCA GAT CTC TAC GCA TTT CAC 3′) for the lower strand. Primers JC254E and JC254F are partially complementary (indicated by boldface type). The second step was PCR amplification with primers JC254A and JC254B and a mixture of fragments A and B as the DNA template. The resulting PCR fragment containing *rrnB* P1 was purified, cut with the NgoMIV and BglII restriction enzymes (restriction sites for these enzymes are underlined in the sequences above), and ligated with previously digested plasmid pNO1301. Similarly, plasmid pDJ2754-15 containing a PCR fragment with the *rrnB* P2 promoter was obtained by a single PCR using oligonucleotides JC254C (5′ ATT CTG ACT GTA GCC GGC ACT GAC ACG GAA CAA CGG C 3′) and JC254B, and plasmid pDJ2754-11 containing a promoterless PCR fragment was obtained by PCR using oligonucleotides JC254D (5′ ATT CTG ACT GTA GCC GGC AAA TTG AAG AGT TTG ATC ATG GC 3′) and JC254B.

To clone different lengths of the *rrnB* operon, we first generated a pKK223-3 (Pharmacia) derivative designated pDJ2790 that lacked the *tac* promoter by deleting a BamHI fragment containing the promoter. Vector pDJ2790 was then digested with the BamHI and PstI restriction enzymes and ligated with BamHI-PstI-digested PCR fragments containing different lengths of the *rrnB* operon. Plasmid pDJ2791A contained a PCR fragment which was obtained in a reaction using oligonucleotides JC291A (5′ GCC GGC GAT TAA GGA TTC TGC ACG TCT GAC GGC AAA TGG 3′) and JC291B (5′ CTT TCT ATC AGA CTG CAG GTG TGA GCA CTA CAA AGT ACG C 3′) (the recognition sites of the BamHI and PstI restriction enzymes are underlined). The pDJ2791B plasmid contained a PCR fragment that was obtained in a reaction using oligonucleotides JC291A and JC291C (5′ GAG CGT CAG TCT CTG CAG AGG GGG CCG CCT TCG CCA CCG G 3′).

Plasmid pDJ2845 was obtained by cloning a PCR product that encompassed the sequences immediately downstream of *rrnB* P2 to the end of the gene encoding the 16S rRNA into the PstI sites of pKK223-1. The oligonucleotides used to synthesize the DNA fragment were JC345A (5′ ATA AAT TTA ATA CTG CAG CCG CGC CGC TGA GAA AAA GCG 3′) and JC291A. The proper orientation of the cloned fragment was confirmed by restriction enzyme mapping.

Microscopy and image analysis. All samples used for microscopy were fixed with formaldehyde. To fix the cells, 5-ml aliquots of cultures were removed from the culture flasks, and formaldehyde was added to a final concentration of 3.7%. Cells were fixed for 1 h at room temperature, centrifuged, and resuspended in 1 ml of 1× phosphate-buffered saline. Before the cell mixtures were mounted on slides, 15 μl of a 10-μg/ml solution of 4′,6′-diamidino-2-phenylindole (DAPI) was added to each mixture. The final mixtures of fixed cells were mounted on slides using 1% low-melting-point agarose. Microscopy was performed with a Zeiss Axiophot II microscope equipped with a Plan-Apo ×100 objective, epifluorescence filters, and a 2.5 optovar. Images were captured with a charge-coupled device camera (Micromax) working at 2X2 binning. The images were processed with Adobe Photoshop.

The percentage of RNAP-GFP fluorescence emitted from the cytoplasm in individual cells was calculated by adding the intensities of all the pixels corresponding to the nucleoid-free area, dividing the number obtained by the sum of the intensities of all the pixels of the cell, and then multiplying the result by 100. The background intensity, measured in cell-free areas of the micrograph, was subtracted during the calculations. The measurements were obtained using digital pictures of 100 different cells for each plasmid-carrying strain, and the values shown in Table 2 are averages and standard deviations. Pixel intensities were measured using IPLab 3.6 (Innovision). The percentage for the RNAP-GFP signal emitted from the cytoplasm of cells without a plasmid was about 15%, which represented either emission from β′-GFP molecules not associated with the nucleoid or just scattering of light from the nucleoid due to the small size of the cells and the limited resolution of the optical microscope.

The transcription foci in the nucleoid were operationally defined as regions of the cell or nucleoid that contained RNAP-GFP signals whose intensity was at least 80% the intensity of the brightest pixel with areas covering less than 10% of the total area (measured in pixels). Because of the small size of the cytoplasmic space, it was very difficult technically to perform the previously described contrast analysis (6) for the RNAP-GFP concentration outside the nucleoid in images of cells carrying plasmids, and the results were not reliable.

TABLE 2. Plasmid construct with *rrn*, growth rate reduction, RNAP distribution, and cytoplasmic RNAP-GFP signal

Plasmid	*rrnB* (transcript length [kb])[a]	Growth rate reduction	RNAP distribution[b]	Cytoplasmic RNAP-GFP signal (%)[c]
pBR322	None	No		17 ± 7
pNO1301	*rrnB* P1-P2-16S-tRNAs-23S-5S (5.4)	Yes		53 ± 4
pNO1302	*rrnB* P1-P2-16SΔ-Δ23S-5S (2.7)	Yes		57 ± 7
pDJ2791A	*rrnB* P1-P2-16S (1.8)	Yes		32 ± 7
pDJ2791B	*rrnB* P1-P2-16SΔ (1.0)	No		15 ± 6
pDJ2754-11	*rrnB* P1-P2Δ 16S-tRNAs-23S-5S (0)	No		14 ± 5
pDJ2754-17	*rrnB* P1-16S-tRNAs-23S-5S (5.4)	Yes		52 ± 3
pDJ2754-15	*rrnB* P2-16S-tRNAs-23S-5S (5.4)	Yes		48 ± 6
pDJ2845	P*tac*-16S (1.8)	No		16 ± 6

[a] With the exception of pBR322, all constructs have the antitermination sequence *boxA* (2, 12) and two *rrn* terminators.

[b] The cell wall is represented by a rounded rectangle, and the nucleoid is represented by an oval. The cytoplasmic space is between the cell wall and the nucleoid of the cell. Gray shading indicates the presence of RNAP-GFP fluorescence signal. When RNAP was detected in the ctyplasmic space, the transcription foci in the nucleoid were diminished.

[c] The cytoplasmic RNAP-GFP signal is expressed as a percentage, which was determined by dividing the RNAP-GFP fluorescence in the cytoplasmic space by the fluorescence in the whole cell, as described in Materials and Methods. The values are averages ± standard deviations for analyses of 100 cells.

RESULTS

Expression of extrachromosomal copies of an *rrn* operon is detrimental to cell growth. To study whether transcription of an *rrn* operon in *trans* affects the location and/or distribution of RNAP, the pBR322-based plasmid pNO1301, which carried an intact copy of the *rrnB* operon, including its two promoters, P1 and P2, and terminators, was introduced into DJ2735 cells by transformation. DJ2735 contains a functional *rpoC-gfp* allele as the sole source of β′ subunits, which allows tracking of RNAP by fluorescence microscopy (6). Intriguingly, two distinct sizes of colonies of Amp^r transformants were observed after overnight growth (about 14 h); approximately 70% of the colonies were small, while the remaining 30% were large (Fig. 1A), suggesting that the population was heterogeneous. Indeed, the doubling times for cells from the large and small colonies when they were grown in LB medium containing ampicillin were 47 and 58 min, respectively (Fig. 1B). Note that the doubling time for cells from large colonies was the same as the doubling time for cells harboring the empty pBR322 vector. Restriction mapping of plasmid DNA isolated from the colonies that were two different sizes revealed that while the plasmids from the small colonies had an intact *rrnB* operon, all of the plasmids from the large colonies had either deletions or rearrangements of the operon (data not shown). As this phenotype has not been described previously, we performed the same experiments with MG1655 cells, and the same results were obtained. Thus, these results demonstrate that expression of extra plasmid-borne copies of the *rrnB* operon is harmful to a cell.

Reasoning that reduced rRNA transcription from both chromosomal and plasmid-borne operons might eliminate the detrimental effect as *rrn* promoter activity is sensitive to nutrient richness (21), we selected for transformants on minimal glucose-ampicillin plates. Indeed, the sizes of the colonies on minimal glucose-ampicillin plates were similar, and the organisms had intact pNO1301 plasmids (data not shown). This indicates that the regulation of the plasmid-borne *rrnB* operon is the same as the regulation in the chromosome. Thus, in all experiments described below, starting cultures of DJ2735 containing pNO1301 or its derivatives were grown initially in minimal glucose medium in the presence of ampicillin to ensure the integrity of the *rrnB* construct.

RNAP localizes in the cytoplasm of cells harboring a plasmid with the *rrnB* operon. We examined the effect of pNO1301 on the location and distribution of RNAP under two different growth conditions (Fig. 2). When cells containing pNO1301 were grown in a nutrient-poor minimal glucose medium, the vast majority of RNAP was located in the nucleoid, and very little of the RNAP-GFP signal was detected in the cytoplasm (Fig. 2A, B, and C). In addition, the transcription foci under these conditions were less evident, as expected (6). Intriguingly, when the cells were grown in nutrient-rich LB medium, a significant amount of RNAP was observed in the cytoplasm in addition to the nucleoid (Fig. 2D to L). In contrast, RNAP was located exclusively in the nucleoids of cells transformed with pBR322 (Fig. 2M, N and O), just like RNAP in cells in the absence of a plasmid, as shown previously (6). Furthermore, while in rich media transcription foci were obvious in the nucleoids of cells harboring pBR322, there were fewer transcription foci in cells containing pNO1301 in these media. The difference in the intensities of the transcription foci in the

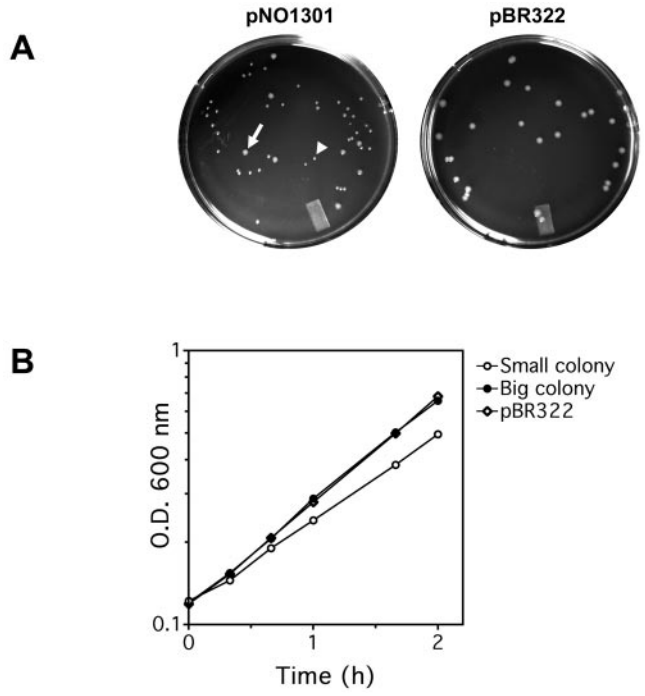

FIG. 1. Presence of plasmid-borne *rrnB* (pNO1301) is detrimental to cell growth in rich media. (A) Heterogeneous colony sizes for cells transformed with plasmid pNO1301 compared to cells transformed with plasmid pBR322 on LB medium plates containing ampicillin. The arrow indicates a representative big colony that emerged in the otherwise small-colony background. A representative small colony is indicated by an arrowhead. (B) Growth curves for big and small transformants with plasmid pNO1301. For comparison, the growth curve for a transformant with pBR322 is included. Growth was monitored in cultures grown in LB medium containing ampicillin at 37°C. O.D. 600 nm, optical density at 600 nm.

nucleoids was evident when the two kinds of cells (cells containing pNO1301 and cells containing pBR322) were mixed prior to examination in the same microscope field (Fig. 2P, Q, and R). For the majority of cells, the RNAP-GFP signals recruited into the cytoplasmic space by plasmid-borne *rrn* operons were diffuse; however, about 8% of the cells containing pNO1301 showed aggregated RNAP-GFP signals in the cytoplasm (Fig. 2E, H, and Q), probably because of "clustering" of plasmids in the cell (9, 22). These results suggest that active transcription of a plasmid-borne *rrn* operon is able to recruit a significant amount of RNAP molecules.

Additionally, a significant percentage (about 13%) of cells harboring pNO1301 were morphologically different than the normal rod-like cells, as shown in Fig. 2G and J. Along an imaginary axis connecting the cell poles, it was apparent that some pNO1301-containing cells were wider than wild-type cells. At present, we do not know the reasons for this phenotype; however, one possibility is that it was caused by extra rRNA made from additional plasmid-borne rRNA operons (25).

Localization of RNAP in the cytoplasm depends on the length of the plasmid-borne *rrnB* transcript but not on its integrity. The complete *rrnB* operon in pNO1301 encodes a 5.4-kb transcript consisting of 16S, 5S, and 23S rRNAs plus a spacer tRNA (Table 2). To determine if transcription of the entire ribosomal operon is required to recruit RNAP molecules outside the nucleoid, a series of pNO1301 derivatives in which different portions of the *rrnB* operon were deleted were examined (Fig. 3 and Table 2).

pNO1302 was a derivative in which a 2.5-kbp SalI fragment encompassing approximately one-half of the 16S and 23S RNA-encoding genes, as well as the whole gene for the spacer tRNA, was removed. This resulted in a 2.7-kb *rrnB* transcript. Similar to the presence of pNO1301, the presence of pNO1302 was detrimental to cells grown in rich LB medium (data not shown). Intriguingly, cells harboring pNO1302 had a much lower growth rate (doubling time, 108 min) than cells containing either the pBR322 control (doubling time, 47 min) or pNO1301 (doubling time, 58 min) in LB broth, suggesting that the synthesis of a partially deleted *rrnB* transcript causes extra stress to the cells. Furthermore, a significant amount of RNAP was also localized in the cytoplasm of pNO1302-containing cells, similar to the effect of pNO1301 (Fig. 3A, B, and C).

Plasmid pDJ2791A, in which only the 16S RNA transcript (1.8 kb) (Table 2) was synthesized, yielded results similar to those obtained with pNO1301 and pNO1302 (Fig. 3D, E, and F) and caused a slow-growth phenotype. In contrast, cells harboring pDJ2791B, which expressed only approximately 1.0 kb of the 16S RNA gene (Table 2), were found to contain RNAP exclusively in the nucleoid (Fig. 3G, H, and I). Furthermore, pDJ2791B was stably maintained in the cells and was not detrimental to cell growth.

We observed a correlation between the length of the plasmid-borne synthesized transcript and the RNAP-GFP signal measured in the cytoplasm of cells carrying the plasmids (Table 2). In cells carrying plasmids that synthesized 5.4- and 2.7-kb transcripts more than 50% of the total cellular RNAP-GFP fluorescence signal was localized in the cytoplasm (plasmids pNO1301 and pNO1302) (Table 2). Cells carrying plasmids that synthesized shorter transcripts had weaker RNAP-GFP fluorescence signals in their cytoplasm, >30% for plasmid pDJ2791A (1.8-kb transcript) and <20% for plasmid pDJ2791B (1.0-kb transcript). In control cells carrying pBR322 or no plasmid at all less than 20% of the total cellular RNAP-GFP fluorescence signal was localized in the cytoplasm. This background signal likely resulted from scattering of light emitted from the nucleoid-bound RNAP-GFP, due to both the small size of the cell and the limited resolution of the optical microscope. We concluded from these experiments that synthesis of an *rrnB* transcript that is a certain length in *trans* is required to recruit RNAP into the cytoplasm.

Localization of RNAP in the cytoplasm depends on active transcription of a plasmid-borne *rrnB* operon. There are two promoters, *rrnB* P1 and *rrnB* P2, for the *rrnB* operon in pNO1301. To determine if either *rrnB* promoter alone is sufficient for cytoplasmic localization of RNAP, a series of pNO1301 derivatives were constructed in which one or both of the promoters were removed (Table 2). In cells transformed with a derivative lacking both *rrnB* P1 and *rrnB* P2 (plasmid pDJ2754-11), no RNAP-GFP signal above the background level was detected in the cytoplasm (Table 2), and all RNAP was localized exclusively in the nucleoid (Fig. 4A, B, and C), similar to the results for pBR322-containing cells. However, in cells containing derivatives that had either only *rrnB* P1 (plasmid pDJ2754-17) or only *rrnB* P2 (plasmid pDJ2754-15), the RNAP-GFP signals were detected in both the cytoplasm and

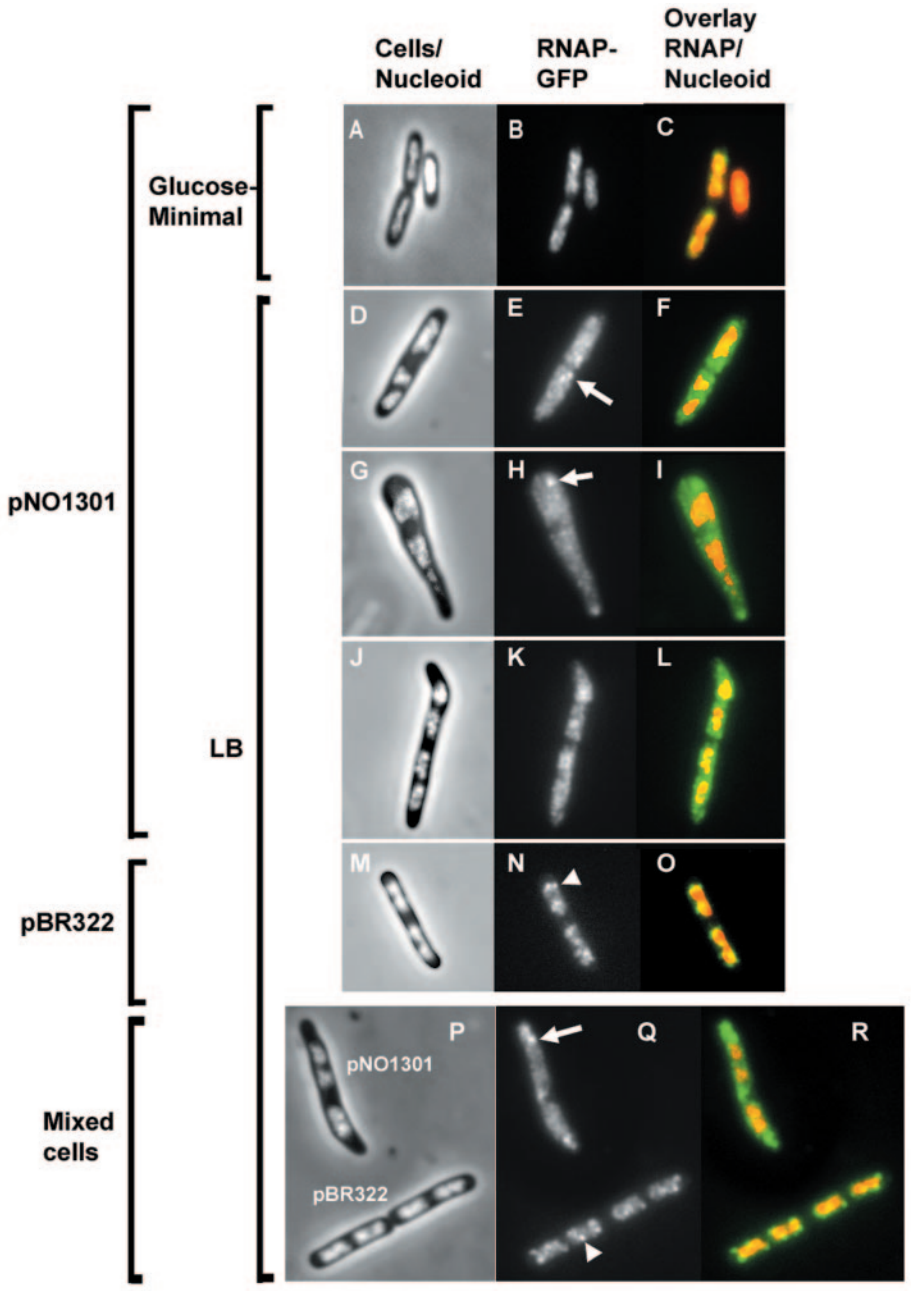

FIG. 2. Cell morphology and location and distribution of RNA polymerase in cells carrying the plasmid-borne *rrnB* operon in pNO1301 grown in rich and minimal media. (A, D, G, J, M, and P) Merged phase-contrast and DAPI fluorescence images. (B, E, H, K, N, and Q) Fluorescence from the RNAP-GFP fusion protein. (C, F, I, L, O, and R) Overlays of the fluorescence from the RNAP-GFP fusion protein (green) and the fluorescence of the DAPI-stained nucleoid (red). (P, Q, and R) Comparison of cell morphology and location and distribution of RNA polymerase in cells carrying either pNO1301 or pBR322 in the same microscope field. Cells carrying pNO1301 and pBR322 were grown in LB medium and mixed at a ratio of 1:1 before fixation, and this was followed by fluorescence microscopy. Representative images are shown. Note that while transcription foci are evident in the nucleoids of cells harboring pBR322 (arrowhead), they are diminished in the nucleoids of cells harboring pNO1301. Although most of the RNAP-GFP fluorescence signals in the cytoplasm are diffuse, sometimes concentrated signals are apparent in the cytoplasm of cells harboring pNO1301 (arrows). For simplicity, only one representative focus is indicated.

the nucleoid (Fig. 4D to I). Image analysis of the micrographs showed that either *rrnB* P1or *rrnB* P2 was able to recruit about one-half of the total RNAP-GFP into the cytoplasm (Table 2). We concluded from these experiments that active transcription of a plasmid-borne *rrnB* operon from either *rrnB* P1 or *rrnB* P2 is sufficient to recruit RNAP molecules from the nucleoid into the cytoplasm.

To determine whether any strong promoter had the same effect as the ribosomal promoters, we replaced both *rrnB* promoters in pDJ2791A with the P*tac* promoter, resulting in plas-

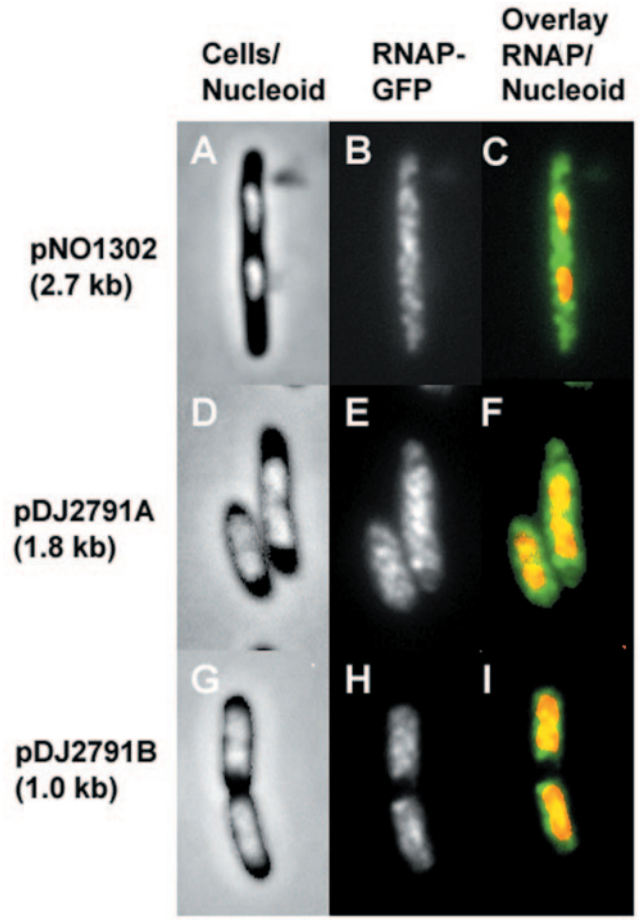

FIG. 3. Cell morphology and location and distribution of RNA polymerase in cells carrying plasmid-borne *rrnB* operons that synthesize transcripts that are different lengths. (A, B, and C) Plasmid pNO1302 (2.67 kb). (D, E, and F) Plasmid pDJ2791A (1.8 kb). (G, H, and I) Plasmid pDJ2791B (1.0 kb). (A, D, and G) Merged phase-contrast and DAPI fluorescence images. (B, E, and F) Fluorescence from the RNAP-GFP fusion protein. (C, F, and I) Overlays of the fluorescence from the RNAP-GFP fusion protein (green) and the fluorescence of the DAPI-stained nucleoids (red).

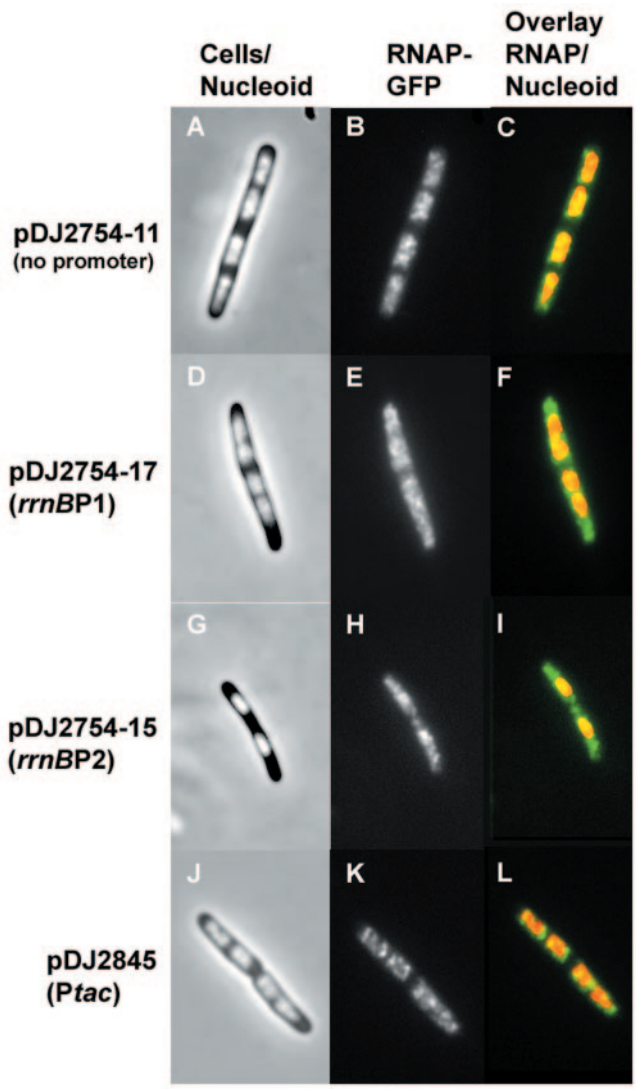

FIG. 4. Cell morphology and location and distribution of RNA polymerase in cells carrying a plasmid-borne fragment of the *rrnB* operon transcribed from different promoters. (A, B, and C) Plasmid pDJ2754-11 (no promoter). (D, E, and F) Plasmid pDJ2754-17 (*rrnB* P1 promoter). (G, H, and I) Plasmid pDJ2754-15 (*rrnB* P2 promoter). (J, K, and L) Plasmid pDJ2845 (*tac* promoter). (A, D, G, and J) Merged phase-contrast and DAPI fluorescence images. (B, E, H, and K) Fluorescence from the RNAP-GFP fusion protein. (C, F, I, and L) Overlays of the fluorescence from the RNAP-GFP fusion protein (green) and the fluorescence of the DAPI-stained nucleoids (red).

mid pDJ2845. In contrast to the results obtained with pDJ2791A, RNAP-GFP was located exclusively in the nucleoids of cells containing pDJ2845 (Fig. 4J, K and L), and there were no cytoplasmic RNAP-GFP signals above the background level (Table 2). This result supports the hypotheis that rRNA transcription from ribosomal promoters in *trans* is uniquely able to recruit RNAP from the nucleoid into the cytoplasm.

DISCUSSION

In this study, we determined the effect of plasmid-borne *rrnB* on the location and distribution of RNAP in the cell. Our results provide direct visual evidence that active transcription of *rrnB* recruits a large amount of RNAP molecules. In cells without extrachromosomal copies of *rrnB*, RNAP is located exclusively in the nucleoid. However, in the presence of a plasmid-borne *rrnB*, RNAP is found both in the nucleoid and in the cytoplasm under some conditions. Also, we demonstrated that caution should be used to ensure the integrity of a plasmid-borne rRNA operon during experiments as active synthesis of extrachromosomal rRNA is detrimental to the cell. Our results are summarized in Table 2.

For RNAP to be recruited into the cytoplasm, two factors are required, as determined in this study. First, *rrnB* transcription must result from the *rrnB* P1 and/or *rrnB* P2 promoter. Furthermore, the effect is evident only when the *rrnB* promoters are fully active, such as during growth in rich medium, and is absent when cells are grown in a minimal medium in which rRNA expression is dramatically reduced. Also, another strong

promoter, P*tac*, was not able to replace the *rrnB* promoters, which is consistent with the extraordinary strength of the ribosomal promoters during rapid growth (12). Second, some minimal length of the *rrnB* transcript, but not necessarily an intact *rrnB* gene, must be present in the plasmid. One possibility is that the DNA sequence encoding the minimal length of the *rrnB* transcript contains necessary regulatory sites for the synthesis of rRNA. Alternatively, this requirement likely reflects the limitation of detection of the RNAP-GFP signals in our system. Assuming that an elongating RNAP molecule covers approximately 85 bp of DNA, as reported by French and Miller (10), at most about 21 RNAP-GFP molecules transcribe the 1.8-kb *rrnB* fragment in pDJ2791A, which is the minimal transcript size with which the effect was observed. However, the RNAP-GFP molecules transcribing a plasmid-borne *rrnB* are unlikely to be saturating (or maximal) as RNAP is probably limiting in the cell, as discussed below. Together, the results of this study demonstrate that active synthesis of ribosomal operons from rRNA promoters is a major driving force for the distribution of RNAP in *E. coli*. Our results are consistent with the results of numerous previous studies which showed that most RNAP molecules engage in rRNA synthesis in optimal growth conditions (4, 21).

Plasmids pNO1301 and pNO1302 were used previously to study rRNA regulation (14), and it was found that synthesis of chromosomal rRNA was reduced by pNO1301 (which contains an intact *rrnB* gene) but not by pNO1302 (which contains an *rrnB* gene with a partial deletion). Based on these results, Jinks-Robertson et al. proposed a "feedback" regulatory mechanism for rRNA expression by free rRNA. Also, it has been reported that fewer RNAP molecules transcribe the chromosomal *rrn* operons in cells harboring pNO1301 than in cells containing pBR322 or pNO1302 (27). While plasmids pNO1301 and pNO1302 had different effects in the previous studies, in our studies these plasmids behaved similarly in two respects, (i) recruiting RNAP into the cytoplasm and (ii) concomitantly decreasing the number of transcription foci in the nucleoid (Fig. 2Q and 3B). Thus, our results suggest that similar to chromosomal rRNA synthesis in cells harboring pNO1301, chromosomal rRNA synthesis in cells harboring pNO1302 is reduced also. The reasons for the apparent difference between the behavior of the two plasmids in our study and the behavior of the plasmids in the other studies mentioned above are not clear at present. One possibility is that the assays and conditions were different in the different studies.

Our study also has implications for cell growth and global gene regulation. Evidently, synthesis of rRNA from extrachromosomal copies of *rrnB* is a burden for the cell, and it could account for about 75% of the total rRNA in the cell (13, 19, 25, 26). The extra, plasmid-borne *rrn* transcripts (either intact or with partial deletions) could affect ribosomal protein synthesis and ribosome assembly, which in turn could disturb the balance of cell growth, leading ultimately to a reduced growth rate. However, another simple explanation is that when RNAP was diverted from the nucleoid into the cytoplasm in the presence of a plasmid-borne *rrnB* gene, the growth rate of a culture in rich medium was reduced dramatically. The complete correlation between the ability of a plasmid-borne *rrn* operon (either intact or with a partial deletion) to recruit RNAP into the cytoplasm and the ability to cause a reduced growth rate is striking (Table 2). This result is consistent with the notion that RNAP is limiting in the cell. Due to recruitment of RNAP into the cytoplasmic *rrn* operons, not only is the synthesis of chromosomal *rrn* operons reduced, as manifested by the decreased number of transcription foci in the nucleoid, but also the expression of other crucial genes that are sensitive to the concentration of RNAP is probably decreased. These combined effects are probably responsible for the reduced growth rate, the formation of small colonies, and the strong selective pressure to mutate the extrachromosomal element. The proposition that the amount of RNAP is limiting in *E. coli* and consequently its distribution and redistribution in the genome is sensitive to changing physiological conditions has been proposed to be an important element in global gene regulation, including the stringent (nutrient starvation) response and the carbon source limiting response (1, 16, 29).

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