JOURNAL OF BACTERIOLOGY, July 1995, p. 3793–3800 Vol. 177, No. 13
0021-9193/95/$04.00+0

Point Mutations in the Leader boxA of a Plasmid-Encoded *Escherichia coli rrnB* Operon Cause Defective Antitermination In Vivo

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Received 3 August 1994/Accepted 8 February 1995

We have introduced point mutations into the leader boxA of a plasmid-encoded *Escherichia coli rrnB* operon to study the in vivo role of this regulatory element in the natural context of rRNA synthesis. The same mutations were previously shown to cause severe antitermination defects in vitro and in the context of a reporter gene assay. The plasmid-encoded *rrnB* mutant constructs studied here also contained point mutations in the 16S and 23S rRNA genes, which were used to distinguish rRNAs derived from plasmid and chromosomal *rrn* operons by primer extension analysis. Point mutations in boxA reduced the fraction of plasmid-derived rRNA in the cell from 75% to about 50%. The reduction was similar for both 30S and 50S subunits as well as 70S ribosomes, suggesting that no transcriptional polarity occurred between the expression of the 16S and 23S rRNA genes in plasmid *rrnB* operons carrying a mutant boxA. The boxA mutations do not affect the amount of transcription initiation, suggesting that a suboptimal leader boxA causes premature transcription termination at an early stage of transcription. Our results are consistent with a role for antitermination in the completion of full-length *rrn* transcripts but give no indications of posttranscriptional boxA functions.

Transcriptional antitermination is the term applied to the postinitiation modification of RNA polymerase such that it no longer recognizes termination signals. To date, the best-characterized general antitermination mechanisms are in the viral systems (for a review, see reference 16). Bacteriophage λ uses two antitermination systems; the human immunodeficiency virus uses yet another (5, 15, 20, 29, 38). A more specialized antitermination mechanism is used in the *Escherichia coli bgl* operon, in which the binding of a protein known as BglG to a specific sequence on the nascent RNA sterically hinders the formation of a terminator structure (3, 4, 21). The first of the λ systems controls the expression of the delayed early genes and depends on the λ *N* gene product, while the second system regulates the expression of the late genes by means of the λ Q protein (47). The N-mediated antitermination system requires a sequence element known as the *nut* (for N utilization) site and several *E. coli* proteins. The host proteins required are known as Nus (for N utilization substance) factors and include NusA, NusB, NusE, and NusG (31). NusE is the ribosomal protein S10. Transcription of the *nut* site induces the formation of a large termination-resistant transcription complex in which the *nut* site RNA, the four Nus factors, the λ N protein, and RNA polymerase are assembled together (20, 35). This transcription complex is very stable and can read though terminators many kilobases from the *nut* site. The *nut* site itself comprises two elements: a ca. 12-nucleotide (nt) boxA sequence, which is conserved among all lambdoid phages (33), and a hairpin boxB motif (12).

The leader regions and intergenic spacer regions of rRNA operons in many eubacteria also have a conserved boxA sequence element, whose core sequence (6 to 11 nt) is similar to the boxA element found in the *nut* sites of phage λ (6, 18, 19, 33). A conserved boxB-like hairpin precedes, rather than follows, the boxA sequence in the leader regions of eubacterial rRNA operons. These similarities and the observation that the *E. coli nusB5* mutation affects λ N-mediated antitermination as well as rRNA synthesis (13, 40) suggest that *E. coli rrn* operons might be subject to a similar antitermination mechanism, although an N-like protein has not yet been found in *E. coli*. In support of this idea, biochemical evidence has shown that NusA, NusB, NusE, and NusG are also likely to play a role in the ribosomal antitermination system (26, 30, 42). In addition, band shift analysis has indicated that NusE and NusB heterodimers specifically bind the ribosomal boxA sequence (36). However, there are some qualitative differences between the two systems; ribosomal antitermination requires at least one extra, as yet uncharacterized, cellular component (possibly an N analog) not required by λ (42), and NusB-NusE heterodimers (32) do not appear to bind λ boxA, at least within the limitations of the gel shift assay (36).

In earlier studies, a 67-bp leader fragment was shown to be sufficient for ribosomal antitermination when inserted between the *E. coli rrnG* P_2 promoter and a Rho-dependent terminator followed by a chloramphenicol acetyltransferase reporter gene (27). A comprehensive mutational analysis of this fragment showed that most of the point mutations that dramatically reduced chloramphenicol acetyltransferase expression in this system reside in the boxA element (6). In another study, in which a plasmid-encoded *rrnB* operon was fused to the λ p_L promoter, the deletion of boxA and sequences further downstream led to reduced expression of the plasmid-encoded operon and a compensatory derepression of the chromosomal copies, indicating that boxA might be important for *rrn* transcription (14). Theissen et al. (43) have replaced the boxA sequence TGCTCTTTAACA with two consecutive hexanucleotide palindromes, also on plasmid-borne operons. This substitution caused decreased growth rates, a reduction in plasmid

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copy number, and reduced amounts of 30S relative to 50S subunits in exponentially growing cells. These results provided evidence for a posttranscriptional role of boxA in ribosome biogenesis. However, deletion or replacement of the entire boxA sequence may change spatial arrangements within the nascent transcript and may affect transcript stability, transcript processing, or early assembly events by virtue of grossly perturbed RNA folding.

To study the specific role of boxA and to minimize potential disruptions of RNA structures and processing events, we have analyzed the effect of two boxA point mutations in the natural context of rRNA transcription and ribosome synthesis. The two mutations (mutants 533 and 553 in reference 6), which were highly defective (>20-fold) in antitermination of the chloramphenicol acetyltransferase reporter gene system, were introduced into a plasmid-encoded *rrnB* operon. The first of these mutations also caused a strong reduction in transcriptional readthrough of a Rho-dependent terminator in vitro (42). Here we demonstrate that both of these boxA point mutations cause a highly reproducible reduction in the amounts of plasmid-derived 16S and 23S rRNAs relative to those of chromosomal operons. The mutations did not affect the amount of transcription initiating at the plasmid-borne *rrnB* operons, and we did not observe a gradient of expression between the 16S and 23S rRNA genes, suggesting that premature transcription termination in mutant operons occurs early in the transcription of 16S RNA or even during transcription of the leader region. In contrast to the results of a previous study, in which the effect of a deletion-substitution of boxA was studied, we did not observe reductions in growth rate or plasmid copy number, nor could we detect a deficiency in 30S ribosomal subunits in operons containing mutant boxAs. The results obtained in the study described here are discussed in the context of current knowledge of antitermination in *E. coli* *rrn* operons.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* JM109, *e*14$^-$ (*mcrA*) *recA1 endA1 gyrA96* (Nal^r) *thi-1 hsdR17* (r_K^- m_K^+) *supE44 relA1* Δ(*lac-proAB*) [F′ *traD36 proAB laqI*^{q} Δ(*lacZ*)M15], was used as the host strain for plasmids. Cells were grown in LB medium (containing, per liter, 10 g of casein hydrolysate peptone no. 140 [Gibco/BRL], 5 g of yeast extract, and 10 g of NaCl and adjusted to pH 7.5 with NaOH). Plasmid pSTL102 (45) is a derivative of pKK3535 (9) and carries a single base substitution (C to T) at position 1192 in the 16S rRNA gene that confers resistance to spectinomycin, as well as a point mutation (A to G) at position 2058 in the 23S rRNA gene that confers resistance to erythromycin. In the present study, *E. coli* JM109 cells containing plasmid pSTL102, pSTLAT3, or pSTLAT5 were grown exclusively in LB medium supplemented with ampicillin (100 μg/ml) as the only antibiotic.

Mutagenesis of boxA and cloning strategies. boxA point mutations were introduced into a plasmid-encoded *rrnB* operon by site-directed mutagenesis (25). For this purpose, plasmid pC6 (7) was employed; it is identical to pKK3535 (9) but carries the 7,508-bp *Bam*HI insert in the reverse orientation. A 3,241-bp *Bam*HI-*Xba*I fragment, which harbors the P_1/P_2 *rrnB* promoter region and the 16S rRNA gene and which terminates in the spacer $tRNA^{Glu}$ gene, was subcloned into the *Bam*HI and *Xba*I sites of M13 mp18 for mutagenesis. Recombinant M13 mp18 DNAs carrying a mutagenized boxA, as inferred from DNA sequencing, were linearized at the *Kpn*I site within the M13 mp18 multiple cloning site. The *Kpn*I site was blunted with nuclease S1, and inserts were excised by cleavage with *Xba*I and were reintegrated into the unique *Eco*RV site (located in the pBR322 moiety) and *Xba*I site of pC6.

The 2.2-kbp *Bgl*II-*Bam*HI fragments of *rrnB* operons carrying the boxA point mutations 533 and 553 (6) were ligated with the 8-kbp *Bam*HI-*Bgl*II fragment of pSTL102. The resulting plasmids, pSTLAT3 and pSTLAT5, were identical to pSTL102 except for the boxA point mutations (see Fig. 1 and 2).

Determination of relative plasmid copy numbers for plasmids pSTL102, pSTLAT3, and pSTLAT5. *E. coli* JM109 cells harboring plasmid pSTL102, pSTLAT3, or pSTLAT5 were grown to an A_{600} of 0.8. Cells (3-ml suspension) of each strain were mixed on ice with 1.5 ml of *E. coli* JM109 cells carrying a 6-kbp derivative of plasmid pT7T3-19U (Pharmacia). Plasmid DNAs were prepared by the acid-phenol method as described previously (46). After concentration by ethanol precipitation, plasmid DNAs were redissolved in 50 μl of 10 mM Tris-HCl (pH 8.0)–1 mM EDTA–20 μg of DNase-free RNase A per ml, and boxA sequences were verified by dideoxy sequencing. Plasmids were linearized with *Bam*HI, separated on 1% agarose gels, and visualized by ethidium bromide staining. Staining intensities of the plasmid bands were measured on a Fluorimager 575 (Molecular Dynamics) and quantified with the software Image Quant 3.3. Intensity values for bands corresponding to pSTL102, pSTLAT3, or pSTLAT5 were compared after normalization to those for the control plasmid in each preparation.

Ribosome preparation and RNA extraction. For the preparation of ribosomes, *E. coli* JM109 harboring plasmids pSTL102, pSTLAT3, and pSTLAT5 were grown to an A_{600} of 0.8. Subsequent steps were performed at 4°C. Cells were disrupted by grinding with alcoa (11). The lysate obtained from 3 g (wet weight) of cells was suspended in 5 ml of TMA I (30 mM NH_4Cl, 10 mM Tris-HCl [pH 7.4], 10 mM $MgCl_2$, 6 mM β-mercaptoethanol). Cell debris was removed by low-speed centrifugation, and the supernatant was centrifuged overnight at 100,000 × *g*. The pellet containing crude 70S ribosomes was suspended in 0.3 ml of TMA I per g of cells. For the preparation of ribosomal subunits, 100 A_{260} units of 70S ribosomes was loaded on a linear sucrose gradient (10 to 40% sucrose in TMA II [30 mM NH_4Cl, 10 mM Tris-HCl {pH 7.4}, 0.3 mM $MgCl_2$, 6 mM β-mercaptoethanol]). To prepare 70S tight-couple ribosomes (17), 100 A_{260} units of 70S ribosomes was loaded on a 10 to 30% linear sucrose gradient in TMA III (30 mM NH_4Cl, 10 mM Tris-HCl [pH 7.4], 6 mM $MgCl_2$, 6 mM β-mercaptoethanol). Gradients were centrifuged for 16 h at 30,000 × *g*, and fractions of 1.25 ml were collected. Fractions containing 30S, 50S, or 70S tight-couple ribosomes were pooled and dialyzed against TMA I. RNA was prepared by phenol extraction. For this purpose, ribosome suspensions in TMA I were adjusted to 0.1 M sodium acetate, 2 mM EDTA, and 0.1% (wt/vol) sodium dodecyl sulfate (SDS). Extractions were performed with 1 volume of phenol (saturated with 10 mM Tris-HCl [pH 7.4]–2 mM EDTA–0.1 mM sodium acetate–0.1% SDS) and were repeated until the interphase remained clear. Aqueous phases were finally extracted with 1 volume of phenol-chloroform (1:1) and 1 volume of chloroform. RNA was precipitated with ethanol and dissolved in water. For the preparation of total cellular RNA, cells (50-ml culture) were harvested at an A_{600} of 0.8 by low-speed centrifugation at 4°C. The pellet was kept on ice and was resuspended in 600 μl of ice-cold buffer A (20 mM sodium acetate [pH 5.5], 1 mM EDTA, 0.5% SDS). An equal volume of phenol (saturated with buffer A) prewarmed to 60°C was added, and the phases were mixed and kept at 60°C for 5 min. The phases were separated by low-speed centrifugation, and removal of material from the interphase was avoided. The phenol extraction was repeated, and the aqueous supernatant was loaded on a NAP column (Pharmacia Sephadex G-25, equilibrated in H_2O) to remove residual phenol. The total cell RNA was eluted from the column; this was followed by ethanol precipitation, and RNA was resuspended in H_2O.

Primer extension analysis. Analysis of the rRNA was performed by employing the strongly terminated primer extension assay described by Sigmund et al. (41). In the annealing step, 0.1 to 7 μg of RNA (essentially the same results were obtained in this range of RNA concentrations, i.e., under conditions of [RNA] ≥ [primer]) was incubated with 2 × 10^6 cpm (about 0.1 pmol) of the ^{32}P-5′-end-labelled primer in 0.1 M KCl–50 mM Tris-HCl (pH 8.5) for 2 min at 90°C; this was followed by slow cooling to room temperature for 1 h. To 1 μl of the annealing solution, 0.5 μl of the individual deoxynucleoside triphosphate-dideoxynucleoside triphosphate (dNTP-ddNTP) (each 1 mM) mixture, 0.5 μl of Superscript-murine leukemia virus reverse transcriptase (Gibco/BRL; 200 U/μl), and a salt mix were added to yield final concentrations of 15 mM Tris-HCl (pH 8.5), 5 mM KCl, 5 mM $MgCl_2$, 5 mM dithioerythritol, 0.1 mM each dNTP or ddNTP, and 20 U of reverse transcriptase in a volume of 5 μl. Assay mixtures were incubated for 30 min at 50°C, and 0.5 μl of RNase A (1 mg/ml) was added; this was followed by incubation for 10 min at 37°C. Reactions were stopped by adding 5 μl of sample buffer (2.7 M urea, 67% formamide, 1× TBE [90 mM boric acid, 90 mM Tris base, 2 mM EDTA, pH 8.3], 0.1% [wt/vol] each bromophenol blue and xylene cyanol blue). Samples were kept in boiling water for 3 min and loaded on a 12% polyacrylamide–8 M urea gel. Radioactive bands corresponding to the primer and extension products thereof were visualized by autoradiography, excised from the gel, and quantified by scintillation counting. The two primer extension products yielded a total of 5,000 to 10,000 Cerenkov cpm. Essentially the same relative proportions of extension products corresponding to plasmid- and chromosomally derived rRNAs were measured by Phosphorimaging of gels or scanning densitometry of autoradiographs.

RESULTS

The importance of the boxA element in antitermination of rRNA transcription has been well-documented both in vivo and in vitro by using reporter gene fusions (6, 27, 42). We wished to extend these studies to the functional role of boxA in its native context of an intact rRNA operon. To do this, we introduced two boxA point mutations, previously identified as having strong antitermination defects in the in vivo and in vitro assays, into the leader region of a plasmid-borne *rrnB* operon.

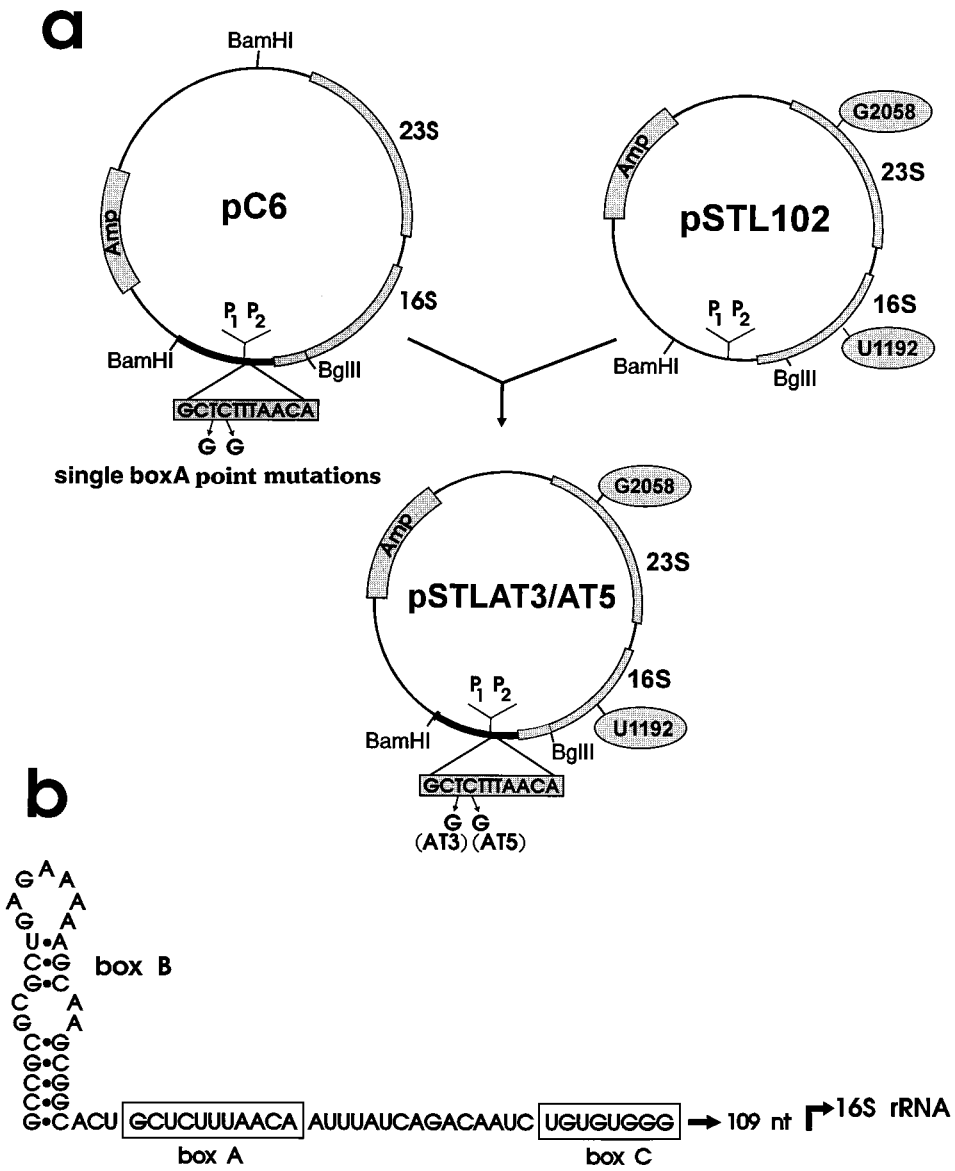

FIG. 1. (a) Insertion of mutant boxA sequences into plasmid pSTL102 (45), yielding plasmids pSTLAT3 and pSTLAT5. Note that pSTL102 has only a single *Bam*HI site. The T→G (pSTLAT3) and C→G (pSTLAT5) substitutions correspond to the boxA mutants 533 (558) and 553 of Berg et al. (6), respectively. (b) Sequence of the *E. coli rrnG* leader transcript downstream of the P_2 promoter (42). boxB denotes the entire hairpin; conserved sequence elements, boxA and boxC, are boxed.

To distinguish between plasmid- and chromosomally derived rRNAs, the plasmid-borne operon also carried the well-characterized spectinomycin and erythromycin resistance mutations in the 16S and 23S rRNA genes, respectively (45). However, cells were grown in the absence of the two antibiotics in order to avoid differences in growth rates due to unequal proportions of resistant ribosomes in cells harboring pSTL102 versus pSTLAT3 and pSTLAT5, and the single point mutations in the 16S and 23S rRNA genes were used only to distinguish rRNAs derived from plasmid and chromosomal *rrn* operons by primer extension analysis. Both of the boxA point mutations studied led to a significant reduction in the amount of plasmid-derived rRNA in the cell, consistent with the importance of the antitermination mechanism in the completion of full-length rRNA transcripts.

Construction of *rrn* operons with mutant boxAs. The construction of plasmids carrying the leader boxA point mutations is described in Materials and Methods and in Fig. 1a. A portion of the *rrnB* operon containing the promoters and the 16S rRNA gene was subcloned into M13 for site-directed mutagenesis. Fragments containing the mutated boxA elements were recloned into a wild-type *rrnB* operon (pC6) for the purposes of some initial experiments and from there were cloned into pSTL102 for the purposes of this study. pSTL102 contains a previously studied *rrnB* operon carrying the spectinomycin and erythromycin resistance mutations at positions 1192 and 2058 of the 16S and 23S rRNA genes, respectively (45). The boxA mutations of the resulting plasmids, pSTLAT3 and pSTLAT5, were confirmed by DNA sequencing (Fig. 2). pSTLAT3 contains a T-to-G mutation in the third nucleotide of the consensus boxA sequence, and pSTLAT5 contains a C-to-G mutation at position four.

Growth rates and plasmid copy numbers. A previous study of a similar nature had shown that a complete replacement of

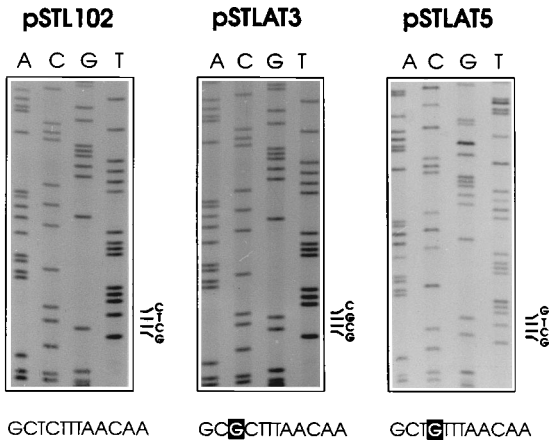

FIG. 2. Dideoxy sequencing of boxA regions of pSTL102 and recombinant plasmids pSTLAT3 and pSTLAT5. Point mutations correspond to nucleotides 1377 and 1378 of *rrnB* according to the numbering system of Brosius et al. (8).

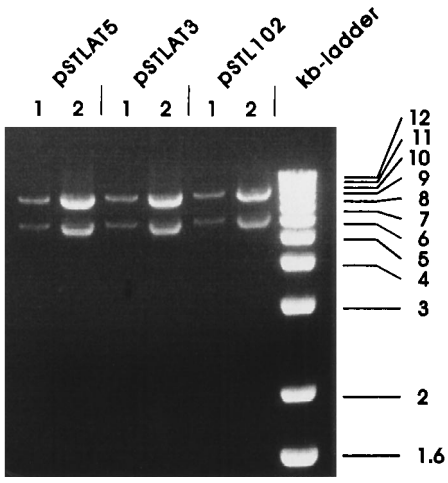

FIG. 3. Relative plasmid copy numbers in JM109 cells harboring plasmid pSTL102, pSTLAT3, or pSTLAT5. Two different dilutions of each plasmid preparation (lanes 1 and 2), linearized with *Bam*HI, were analyzed by electrophoresis in 1% agarose and visualized by ethidium bromide staining. The lower band in each lane corresponds to the 6-kbp reference plasmid used as an internal control to correct for fluctuations of DNA preparations (see Materials and Methods). Intensity values (I_a) (measured by Fluorimaging) of bands corresponding to pSTL102, pSTLAT3, or pSTLAT5 were normalized to those of the control plasmid (I_b) in each lane. I_a/I_b ratios for the three plasmids did not differ by more than ±5%, as confirmed in several independent analyses of the type shown.

boxA with unrelated sequences in the context of a plasmid-encoded *rrnB* operon caused a twofold reduction in plasmid copy number and a 1.05-fold-slower growth rate (43). We therefore measured the growth rates of JM109 cells carrying pSTL102, pSTLAT3, and pSTLAT5 in rich medium at 37°C. We could detect no alteration in the growth rates of strains bearing mutant boxA plasmids (data not shown). We next tested for possible alterations in plasmid copy number. For this purpose, cells containing pSTL102, pSTLAT3, and pSTLAT5 were grown to an A_{600} of 0.8, and identical volumes of each suspension were mixed on ice with a constant volume of reference cells containing a control plasmid to correct for fluctuations of plasmid DNA preparations. Plasmids were linearized with *Bam*HI, and different dilutions of the *Bam*HI digests were analyzed by agarose gel electrophoresis and ethidium bromide staining (Fig. 3). The intensities of bands corresponding to pSTL102, pSTLAT3, or pSTLAT5 were quantified with a Fluorimager and were normalized to those measured for the control plasmid in each preparation (see Materials and Methods). In contrast to the previous study with gross perturbations of the boxA sequence (43), differences in plasmid copy number were insignificant, as inferred from several independent experiments of the kind whose results are shown in Fig. 3. Thus, for the purposes of these experiments, strains carrying pSTL102, pSTLAT3, and pSTLAT5 can be considered physiologically equivalent.

Analysis of rRNAs in ribosomes and ribosomal subunits. Previous studies have indicated that about 70 to 80% of the rRNA in strains carrying an *rrn* operon on a plasmid comes from the plasmid-borne operon (34, 44). We confirmed this for pSTL102, exploiting the point mutations in the 16S and 23S rRNA genes to identify plasmid-derived rRNA. For this purpose, rRNA was analyzed by reverse transcription with 5′-end-labelled primers located just downstream of the mutations. The strategy is described in Fig. 4A and B. Plasmid-derived 16S rRNA gives a primer extension product with an additional 2 nt in the presence of ddATP, by virtue of the C-to-U change at position 1192, whereas chromosomally derived 16S rRNA gives a product with an additional 5 nt. Similarly, plasmid-derived 23S rRNA gives an extension product with an additional 5 nt in the presence of ddTTP, by virtue of the A-to-G change at position 2058, whereas chromosomally derived 23S rRNA extends by only 1 nt.

We first measured the amount of plasmid-derived rRNA in ribosomes and ribosomal subunits from JM109 cells carrying pSTL102 and the two boxA mutant plasmids, pSTLAT3 and pSTLAT5. 30S and 50S subunits and 70S tight-couple ribosomes were isolated from sucrose gradients, and rRNA was extracted (see Materials and Methods). Reverse transcriptase reactions were performed on the isolated RNAs as outlined in Fig. 4A and B, and the extension products were separated on denaturing polyacrylamide gels. An example of such an experiment for the small-subunit rRNA is shown in Fig. 4D. The extension products visualized by autoradiography were excised from the gels and quantified by scintillation counting. The results obtained with rRNAs isolated from 30S and 50S subunits and 70S tight-couple ribosomes are summarized in Table 1. In cells transformed with an *rrnB* operon containing a wild-type boxA, 75% of the rRNA in ribosomes and ribosomal subunits is plasmid derived. However, the proportion of plasmid-derived rRNA in strains carrying an *rrnB* operon with either the pSTLAT3 or the pSTLAT5 mutation is closer to 50%. This indicates either that rRNA is not synthesized efficiently from operons containing boxA mutations or that rRNA derived from mutant operons is functionally defective and is not packaged into ribosomes.

Analysis of rRNA in total cellular RNA. To determine whether the boxA point mutations led to a significant accumulation of functionally defective rRNA, we carried out an analysis of the proportion of plasmid-derived 16S rRNA present in total cellular RNA. The results were the same as those obtained with rRNA isolated from ribosomes or ribosomal subunits; about 75% of the rRNA was derived from plasmids containing *rrn* operons with a wild-type boxA, whereas operons with mutant boxAs contributed only about 50% to the total cellular rRNA (data not shown). Thus, the deficiency caused by boxA mutations appears to be in the synthesis of rRNA and not in its ability to be packaged into ribosomes.

Mapping of the rRNA synthesis defect. To determine where the deficiency in rRNA synthesis occurred, we carried out a

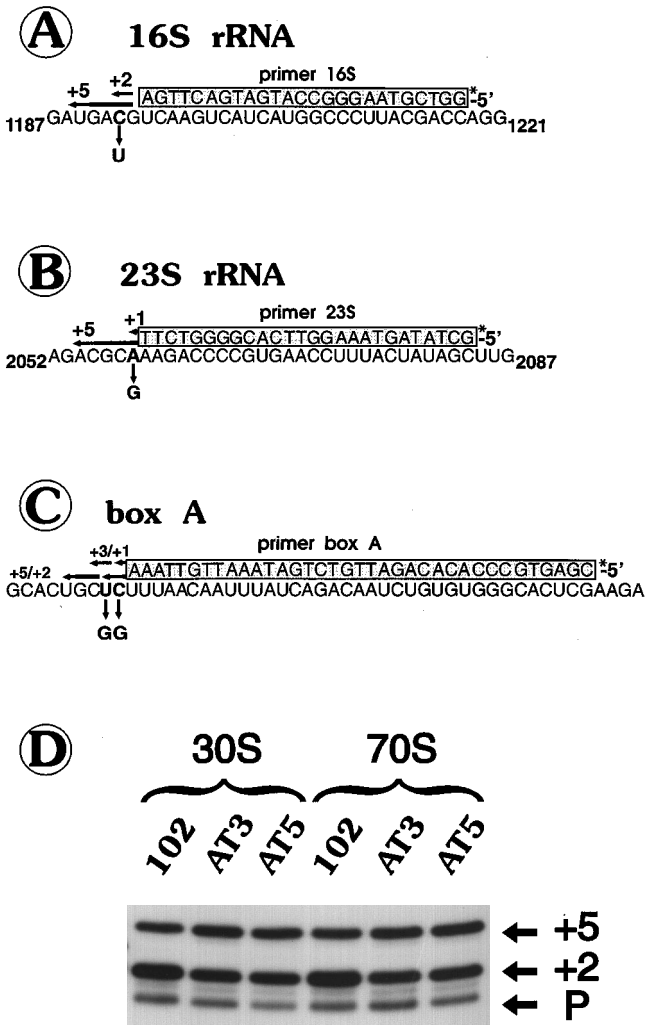

FIG. 4. Primer extension strategies to determine the proportions of plasmid-derived and chromosomally encoded 16S and 23S rRNAs and promoter-proximal transcripts. (A) A ^{32}P-5′-end-labelled primer (25-mer), complementary to nt 1218 to 1194 of mature 16S rRNA, was extended in the presence of ddATP; extension of the primer by 2 nt took place on plasmid-derived 16S rRNA, and extension by 5 nt occurred on chromosomally encoded 16S rRNA. (B) A 26-mer complementary to nt 2084 to 2059 of mature 23S rRNA was extended by 1 nt (chromosomally derived rRNA) or 5 nt (plasmid-derived rRNA) in the presence of ddTTP. (C) Results with a 36-mer complementary to nt 1414 to 1379 of *rrnB* according to the numbering system of Brosius et al. (8). On chromosomally derived transcripts, the primer was extended by 1 nt (ddGTP) or 2 nt (ddATP); on plasmid-derived transcripts, the primer was extended by 3 nt (pSTLAT5) in the presence of ddGTP or 5 nt (pSTLAT3) in the presence of ddATP. For details of the reverse transcription reaction, see Materials and Methods. (D) Separation of primer extension products obtained with the 16S rRNA primer on a 12% polyacrylamide–urea gel. RNAs isolated from 30S subunits and 70S tight-couple ribosomes of *E. coli* JM109 strains harboring plasmids pSTL102, pSTLAT3, and pSTLAT5 were used as templates. Extension products +2 (27 nt) and +5 (30 nt) of the primer (P) (25 nt) are indicated.

similar primer extension analysis closer to the boxA sequence itself. The strategy is described in Fig. 4C. All seven *rrn* operons in *E. coli* have identical sequences in the part of the leader region depicted in Fig. 4C (28). This was exploited to distinguish between *rrn* leader transcripts derived from wild-type chromosomal operons and plasmid operons containing the boxA mutations in pSTLAT3 and pSTLAT5. The results are summarized in Table 1. In this case, the proportion of plasmid-derived leader transcripts from operons with mutant boxAs

TABLE 1. Proportion of plasmid-encoded rRNA in strains carrying various plasmids

RNA[a]	Ribosome[b]	% Plasmid-encoded rRNA[c] in strain carrying:		
		pSTL102	pSTLAT3	pSTLAT5
16S	30S	74 (3)	53 (2)	55 (2)
	70S	77 (3)	57 (4)	56 (5)
23S	50S	77 (5)	58 (7)	56 (7)
	70S	74 (3)	43 (9)	51 (7)
boxA			76 (3)	74 (4)

[a] 16S and 23S, rRNAs detected by the primer extension strategies shown in Fig. 4A and B; boxA, primer extension performed with total cellular RNA according to Fig. 4C.

[b] 30S and 50S, 30S and 50S subunits, respectively; 70S, 70S tight-couple ribosomes.

[c] Values are the averages from six to nine independent experiments; standard deviations are given in parentheses.

was very similar to that of plasmid-derived 16S and 23S rRNAs from wild-type operons, i.e., 75%. Thus, the number of RNA polymerases initiating and transcribing the boxA sequence seems to be unaffected by the presence of these mutations. The simplest interpretation is that RNA polymerase engaged on *rrn* operons harboring mutant boxAs has increased premature transcription termination compared with that engaged on operons with a wild-type leader boxA. If this is so, then termination must take place downstream of the leader primer and upstream of the spectinomycin resistance mutation at position 1192. It is of interest that no further termination appears to occur between the locations of the 16S and 23S primers; i.e., the proportions of plasmid-derived 16S and 23S rRNAs are always the same. Possible reasons for this are discussed below.

DISCUSSION

The function of the boxA element of *E. coli* leader regions has been extensively studied in the context of reporter gene fusions (6, 27). In those experiments, constructs consisted of an *rrn* P_2 promoter, the leader region containing boxA, termination-active sequences, and a promoterless chloramphenicol acetyltransferase gene. A synthetic boxA was also shown to be sufficient for effective readthrough of Rho-dependent terminators, and it was demonstrated by random mutagenesis of the *rrn* leader region that the majority of mutations causing defective antitermination reside in boxA (6). Recently, it has been demonstrated that readthrough of a Rho-dependent terminator in an in vitro transcription assay also depends on a wild-type boxA; constructs lacking boxA or containing a mutant boxA with the same T-to-G substitution as in plasmid pSTLAT3 were unable to promote efficient transcriptional readthrough (42).

In the study reported here, we have analyzed the effects of leader boxA point mutations in the natural context of rRNA transcription, processing, and ribosome assembly. Our results document that the synthesis of rRNA is significantly impaired in *rrn* operons carrying such mutations and suggest that they cause premature transcription termination early in operon transcription. Assuming that plasmid- and chromosomally derived wild-type operons are equally efficient in rRNA synthesis, then some 20 copies of the plasmid-derived *rrnB* operon contribute 75% of the total cellular rRNA and the 7 chromosomal copies produce the remaining 25% (Table 1). The proportion of plasmid-derived rRNA changes to 50% because of the boxA point mutations, but plasmid copy numbers remain unchanged (Fig. 3). Thus, the 20 plasmid operons and the 7 chromosomal

operons each contribute 50% of the rRNA. If one normalizes these values to a single operon, this means that the wild-type boxA promotes the synthesis of full-length transcripts about threefold more efficiently than a mutant boxA. Both of the boxA mutations studied here showed about a 20-fold decrease in terminator readthrough in the reporter gene system (6). Although the effects measured here are not nearly as dramatic, it is likely that the apparent discrepancy can be accounted for by factors such as variations in terminator efficiency or the distance between boxA and the terminator sequence.

The results suggest that the boxA point mutations influenced only transcriptional events. However, we cannot completely rule out the possibility that they might also affect early posttranscriptional events, such as RNA processing and/or assembly, leading to rapid degradation of defective RNA carrying a mutant boxA. However, we consider this possibility unlikely, since the steady-state proportion of leader RNA with a mutant boxA was 75% (Table 1), while that of chromosomally derived wild-type leader RNA was only 25%. This is exactly the same proportion as observed for plasmid-derived 16S and 23S rRNAs in cells containing pSTL102 (wild-type boxA [Table 1]). Thus, our results make it more likely that the antitermination function of boxA can indeed be uncoupled from posttranscriptional events.

The lambdoid phage HK022 blocks superinfection by λ by causing transcription termination at or near the λ *nut* sites. This effect is mediated by the HK022 *nun* gene product. boxA mutations in λ *nutR* which switch the termination function of HK022 Nun protein to that of an antiterminator of transcription have been isolated (39), indicating that the properties of termination and antitermination are closely related. It is thus possible that the ribosomal boxA mutations cause a similar interconversion of function of the transcription complex, in this case from antitermination to termination.

Termination and antitermination may also be explained by kinetic coupling between RNA polymerase and Rho, which predicts that the efficiency of Rho-dependent termination is an inverse function of the elongation rate of RNA polymerase (22). Evidence for the validity of the kinetic coupling mechanism comes from studies of fast- and slowly moving mutant RNA polymerases and a slowly acting mutant Rho protein (22). Recent observations indicate that the ribosomal boxA is responsible for the high rate of transcription elongation of rRNA relative to mRNA (45a). An rRNA operon containing a mutant boxA would thus be predicted to have a slower transcription elongation rate. Thus, it is possible that the proposed premature termination event could be due to a decrease in the transcription elongation rate caused by defective antitermination complexes.

Assuming defective antitermination, the region of premature transcription termination would map to between the two primers used to analyze transcription of the leader boxA and the 16S rRNA gene (Fig. 4). Several groups have identified potential terminators in this region. A similarity between *rrn* leader regions downstream of P_2 and Rho utilization (*rut*) sites was pointed out by Theissen et al. (43). In addition, potential Rho-independent terminator stem-loops have been identified in *E. coli rrn* leader regions (24, 28) and in the *Thermus thermophilus* 16S rRNA leader (18) downstream of boxA and proximal to the 16S rRNA gene start. However, it is unclear whether these latter structures are subject to the antitermination mechanism, which has been shown to function only with Rho-dependent terminators (2). Potential sites for termination in the 16S rRNA gene reside within a 567-bp *Hin*dIII fragment (nt 80 to 647 of the mature *rrnB* 16S rRNA) shown previously to have strong Rho-dependent termination activity in vivo (1).

There was no evident transcriptional polarity between the 16S and 23S rRNAs transcribed from plasmid operons harboring boxA point mutations; i.e., the proportion of plasmid-derived 16S and 23S rRNAs was always the same. Likewise, no transcriptional polarity was observed when boxA, as part of a plasmid-encoded *rrnB* operon, was replaced with 12 nt of unrelated sequence (43). Thus, all of the RNA polymerases that succeed in negotiating the transcription block in the 16S rRNA gene are capable of transcribing to the end of the operon without falling off. This could be explained in several ways. One possibility, although less likely, is that there are no further sites of transcription termination downstream of the 16S rRNA gene primer. Another possibility is that the antitermination complex gets "recharged" by the second boxA element in the spacer region between the 16S and 23S rRNA genes and that all of these RNA polymerases are now capable of transcribing to the end of the operon. Yet another explanation is that a proportion of RNA polymerases are unable to assemble a complete termination-resistant elongation complex because of weaker binding of one or more of the antitermination factors to the mutant leader boxA RNA. However, once an RNA polymerase molecule has succeeded in tethering the entire set of factors, the elongation complex may be persistently stable throughout operon transcription because of its multiple and highly cooperative interactions (31).

Previous studies of the role of boxA in *rrn* transcription involved either the deletion of boxA (14) or its replacement by two hexanucleotide palindromes of unrelated sequence (43). The deletion-substitution of boxA in the latter study led to a reduction in growth rate and plasmid copy number and a deficiency of 16S rRNA and 30S ribosomal subunits versus 23S rRNA and 50S ribosomal subunits. Those observations were interpreted as evidence that boxA has posttranscriptional functions. Since no indication of any of these defects has been found in our study, we propose that the antitermination function of boxA can be uncoupled from effects on processing or assembly. It is possible that replacing boxA by 12 nt of unrelated sequence (43) also removed overlapping or interdigitated sequence elements that are important for ribosome biogenesis, given the proximity of boxA to the processing stalk of the 16S rRNA. Furthermore, the boxA elements of both *rrn* leader regions and λ *nut* sites have been shown to be involved in secondary or higher-order structures when cotranscribed with boxB (35, 37). Thus, it is not surprising that gross perturbations of RNA structure in this region affect the efficient and highly coordinated process of ribosome biogenesis.

As mentioned above, the growth rates of cells harboring the plasmids pSTLAT3 and pSTLAT5 were indistinguishable from those of cells transformed with pSTL102. This suggests that the rates of ribosome synthesis are equivalent in these strains. Since enhanced expression of chromosomal *rrn* operons was observed in strains carrying the mutant operons on plasmids, this suggests that the increased premature transcription termination caused by the boxA mutations is compensated for by enhanced expression of the chromosomal operons. This is consistent with the predictions of the ribosome feedback model of Nomura and coworkers (for a review, see reference 23). The increase in expression in such cases is probably caused by enhanced transcription initiation, which is allowed in part by increased elongation rates and consequently increased promoter clearance (10). In *E. coli nusB5* mutants, which also have defective *rrn* antitermination (40), increased initiation of *rrn* transcription similarly occurs in an attempt to compensate for the decreased processivity. However, in contrast to our results, the ratio of 16S rRNA to 23S rRNA transcripts is increased in *nusB5* strains. It is likely, assuming that NusB associates stably

with the elongation complex as it does in the N-mediated system of λ (31), that a mutant NusB renders the antitermination complex likely to fall apart at any point along the length of the operon. In contrast, the boxA RNA may be only transiently associated with the elongation complex or may become functionally dispensable after assembly of a stable elongation complex including the entire set of Nus factors. This possibility is supported by the observation that certain boxA mutations can be suppressed by the overproduction of NusB (39a), suggesting that once the antitermination complex has been formed with a mutant boxA, it remains stable throughout the length of the operon. Thus, the boxA mutations may simply limit the fraction of RNA polymerases that become modified to a termination-resistant form.

In summary, point mutations in the leader boxA element in rRNA operons decreased the synthetic capacities of those operons. The defect appears to be at the level of premature termination early in operon transcription and not at any subsequent levels of rRNA processing or ribosome assembly. Our results demonstrate clearly the importance of the antitermination mechanism in the faithful and processive synthesis of completed rRNAs in *E. coli.*

ACKNOWLEDGMENTS

We thank Alexander Huttenhofer and Harry F. Noller for providing plasmid pSTL102, Werner Schröder for the synthesis of oligonucleotides, Catherine L. Squires and Wolf-Dietrich Hardt for helpful discussions, and Susanne Parbus and Jochen Heinrich for their help with the Fluorimager measurements.

Financial support for these studies from the Deutsche Forschungsgemeinschaft (SFB344/C2) to R.K.H. and by National Institutes of Health grant GM24751 to Catherine L. Squires is acknowledged.

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