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Characterization of Promoter-Cloning Plasmids: Analysis of Operon Structure in the *rif* Region of *Escherichia coli* and Isolation of an Enhanced Internal Promoter Mutant

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Using the promoter-cloning vehicle described by An and Friesen (J. Bacteriol. **140**:400-410, 1979), *Escherichia coli* chromosomal deoxyribonucleic acid fragments derived from the λ d*rif*^{d}18 transducing phage were cloned in one of several unique restriction endonuclease sites adjacent to tetracycline(*tet*) genes that lack their own promoter. One of these plasmids has been used to isolate nine variants having mutations that lie in a putative internal promoter which is located between *rplL* and *rpoB*. Deoxyribonucleic acid sequence analysis revealed that, in all nine mutants, a single base change, C to T, in the ribonucleic acid polymerase recognition site led to a large increase in promoter activity. Analysis of a variety of plasmids in which *tet* is fused to various promoters yielded the following results: (i) *rplK* and *rplA*, genes for ribosomal protein L11 and L1, respectively, were cotranscribed from a common promoter located upstream from *rplK*; (ii) there was a strong promoter in the region between the *rplKA* operon and *rplJ*, the gene for ribosomal protein L10; (iii) an attenuator region was located between *rplL*, the gene for ribosomal protein L12, and *rpoB*, the gene for ribonucleic acid polymerase subunit β; (iv) transcription terminated immediately after *rpoC*, the gene for ribonucleic acid polymerase subunit β'; (v) a gene coding for unknown protein U, which is located between *tufB* and the *rplKA* operon, had its own promoter; (vi) the *tufB* gene was separated from all of the genes described above and had its own promoter.

We have recently developed a set of plasmids which readily allow the detection of DNA fragments carrying promoters of *Escherichia coli* genes (2). In this communication we demonstrate the usefulness of these plasmids, not only for the detection of promoters, but also for the analysis of operon structure. For this purpose we have chosen to analyze the organization of transcriptional units in the *rif* region at 88 min on the *E. coli* linkage map (4). There is a cluster of four ribosomal protein structural genes, *rplA* (L1), *rplJ* (L10), *rplK* (L11), and *rplL* (L7/L12), and two RNA polymerase subunit genes, *rpoB* (β) and *rpoC* (β') (7, 25, 26, 28, 44-46), in this region. One elongation factor gene, *tufB* (EF-Tu) (19, 20), one rRNA operon (*rrnB*) (24), and a cluster of tRNA genes (45) have also been mapped near the ribosomal protein structural gene cluster. Previous work from several laboratories has suggested that the *rpoBC* genes are cotranscribed with *rplJL* (14, 28, 45) and that the *rplK* and *rplA* genes are cotranscribed independently of the *rplJL-rpoBC* operon (14, 46). In addition, it has been suggested previously that *tufB* is under a separate promoter (27) and that gene *U*, coding for unknown protein P20, has its own promoter (46). Recently, Barry et al. (5) have observed at least two weak promoters in the *rplJL-rpoBC* region in addition to the major promoter, *rplJp*, of this operon. Thus, there exists a large body of information with which to compare the results obtained with our promoter-cloning plasmids. In using these plasmids to verify the basic operon structure outlined above, we have not only demonstrated the power of this method of analysis, but also have added to the knowledge of the genetic organization of this region by isolating and precisely locating a mutant with enhanced activity in an internal promoter of the *rplJL-rpoBC* operon.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *E. coli* K-12 strain JF1161 (*hsdR hsdM*$^{+}$ *lac gal met*) (2) was used as a host for the plasmids throughout these experiments, unless otherwise mentioned. *E. coli* strain JF943 (F^{-} *thi-1, thr-1 leu-6 recA rts rplK rplL gal xyl mtl ara tonA*) was described earlier (14). The maxicell strain *E. coli* CSR603 (*recA1 uvrA6 phr-1*) was from D. Rupp (39). The minicell-producing strain RH17 (*thi leu thr minA minB cycA nal*) was from H. Adler (1). Cells were grown in L-broth (23) in a Gyrotory water bath (New Brunswick Scientific Co.) at 37°C, unless otherwise mentioned.

Plasmid preparation. Plasmids were amplified by

growth, at 37°C, of the culture in L-broth containing 300 μg of spectinomycin per ml (8). Covalently closed circular plasmid DNA was purified by CsCl-ethidium bromide density gradient ultracentrifugation of cleared bacterial lysates (8). For rapid determination and characterization of restriction enzyme digestion patterns, a small amount of DNA was purified as follows: 5-ml cultures were grown in L-broth (23) in 25-ml test tubes. When the absorbance at 600 nm was about 0.6, spectinomycin was added to a final concentration of 300 μg/ml; this was followed by further incubation for at least 16 h. Cells were collected by centrifugation in a table-top Sorvall centrifuge. After the cells were suspended in 50 μl of a solution containing 25% sucrose and 50 mM Tris-hydrochloride, pH 7.5, the mixture was transferred to a 1.5-ml Eppendorf test tube, and 10 μl of a 4-mg/ml lysozyme solution and 15 μl of a 0.25 M EDTA solution were added. After 10 min at 0°C, 60 μl of a solution containing 50 mM Tris-hydrochloride, pH 7.5, 62.5 mM EDTA, and 2% sodium dodecyl sulfate was added, and the mixture was vigorously blended in a Vortex mixer to lyse the cells. After 10 min of incubation at 0°C, 30 μl of 5 M NaCl was added, and the mixture was kept at 0°C for at least 1 h. Most chromosomal DNA and cell debris were removed by centrifuging at 30,000 × *g* for 20 min. The supernatant was transferred to another 1.5-ml test tube; 3 volumes of cold 95% ethanol was added, and the tube was kept at −80°C for 10 min. Plasmid DNA was pelleted by centrifugation in a microcentrifuge for 5 min, and the pellet was dissolved in 200 μl of 0.3 M sodium acetate, pH 7.0. After 600 μl of 95% ethanol was added, the mixture was chilled at −80°C for 10 min. The plasmid DNA was pelleted by centrifugation and dried at 50°C for 10 min. The DNA was suspended in 50 μl of a solution containing 10 mM Tris-hydrochloride, pH 7.5, and 1 mM EDTA. The plasmid DNA prepared by this method was stable for several weeks at 0°C, and sufficient material for restriction endonuclease digestion and transformation was obtained.

Preparation and labeling of maxicells and minicells; polyacrylamide gel electrophoresis. Proteins encoded on a plasmid were radioactively labeled with [^{35}S]methionine in either a maxicell strain or a minicell strain, as described earlier (39), with minor modifications. A 5-ml culture of maxicells that had been transformed with a plasmid, growing in MOPS (morpholinepropanesulfonic acid) medium (32) with 0.2% glucose, was irradiated with UV light from a germicidal lamp (20-cm distance, 36 cm wide) and incubated in a 25-ml test tube overnight at 37°C. After centrifugation and resuspension in fresh medium, the cells were labeled for 30 min with 5 μCi of [^{35}S]methionine (760 Ci/mmol; Amersham Corp.) in 100 μl of MOPS medium containing 0.2% glucose, adenosine, and uracil, each at 20 μg/ml, thiamine at 10 μg/ml, and 19 amino acids lacking methionine, each at 50 μg/ml (3). The expression of proteins from a plasmid in a minicell strain was measured as described earlier (3). Minicell-producing bacteria (RH17) were grown in 200 ml of L-broth to an absorbance at 600 nm of approximately 1.0. The cells were collected and suspended in 40 ml of minicell buffer (50 mM Tris-hydrochloride [pH 7.5], 0.1 M NaCl, 1 mM EDTA). This cell suspension was centrifuged at 3,000 × *g* for 1 min, and the supernatant was centrifuged as described above. The final supernatant from these centrifugations was centrifuged at 10,000 × *g* for 5 min. The pellet was suspended in 1 ml of minicell buffer and layered on a 26-ml, 5 to 30% sucrose gradient in minicell buffer; this was centrifuged at 4,000 × *g* for 15 min in a swinging-bucket rotor (Sorvall). The minicell band was collected, centrifuged at 10,000 × *g* for 5 min, and suspended in minicell buffer. About 10^9 minicells were labeled with 5 μCi of [^{35}S]methionine in 100 μl of MOPS medium as described above. The labeled cells were pelleted and lysed by boiling for 5 min in sodium dodecyl sulfate sample buffer consisting of 125 mM Tris-hydrochloride, pH 6.8, 3% sodium dodecyl sulfate, 10% glycerol, and 5% 2-mercaptoethanol, and the proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (22).

Determination of tetracycline resistance. The tetracycline resistance level was measured by observing survival of colonies on L-broth agar (Difco) containing various concentrations of tetracycline. Cells were grown exponentially in L-broth with 25 μg of chloramphenicol per ml. A small portion of the culture was taken out and streaked on fresh L-broth agar containing 25 μg of chloramphenicol per ml plus various concentrations of tetracycline. After 24 h of incubation at 37°C, survival of the cells was measured by observation of the growth of colonies on the various concentrations of tetracycline. The maximum concentration of the drug that allowed colonies to form was used as a measure of the tetracycline resistance level for any given plasmid-bearing strain. In a previous publication (16) we have reported tetracycline resistance as determined after 12 h of growth on agar at 37°C. Subsequently it has been found that this did not allow detection of weak promoters. Therefore, in the experiments reported here the tetracycline resistance level was read after 24 h of incubation. This difference in incubation time has resulted in slight differences in measured tetracycline resistance at the lower end of the scale, compared with earlier published results (16). The tetracycline agar was prepared as follows: after autoclaving, the L-broth agar was cooled to 50°C and divided into several portions to which an appropriate amount of tetracycline (Sigma Chemical Co.) was added; then the agar was poured into petri plates. The agar was cooled to room temperature immediately and stored at 4°C until use. The tetracycline agar was prepared freshly every day.

Plasmid mutagenesis. A strain carrying pGA78 was incubated in L-broth at 37°C. When the absorbance at 600 nm reached about 0.6, 300 μg of spectinomycin and 10 μg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine per ml were added. After further incubation for about 16 h, the mutagenized plasmid DNA was isolated (2) and transformed into *E. coli* strain JF1161, screening for chloramphenicol resistance.

DNA sequence determination. There is a unique endonuclease *Sal*I site 26 base pairs (bp) upstream from the ATG start codon for *rpoB* (33). After end labeling of this *Sal*I site with [α-^{32}P]dCTP and DNA polymerase large fragment (21), the 499-bp *Eco*RI-*Sal*I fragment carrying the *rplL-rpoB* intercistronic region was purified on a 5% acrylamide gel and was

then eluted from the gel. DNA sequencing was done as described by Maxam and Gilbert (31). For confirmation of this sequence, the DNA sequence of the complementary strand was analyzed by labeling a unique *Taq*I site in the 499-bp *Eco*RI-*Sal*I fragment and sequencing toward *rpoB*.

Other methods. Restriction endonuclease digestion of DNA and DNA ligation were carried out as suggested by the manufacturers (Bethesda Research Laboratories, New England Biolabs, or Boehringer-Mannheim). Transformation of cells was performed as described earlier (30).

RESULTS

Our aims were to demonstrate the usefulness of the promoter-cloning plasmids described by An and Friesen (2) by using them to isolate a promoter mutation and to analyze the operon structure of the *rif* region of *E. coli*. As a background for this, a restriction endonuclease map of λ d*rif*^{d}18 is presented in Fig. 1. The positions of the major promoters (P), possible internal promoters [(P)], an attenuator (At), and a transcription termination signal (T) are indicated. These positions are assigned on the basis of the present and previously published information (14).

Organization of the *rplJL-rpoBC* operon. We have recently shown that the promoter for *rplJ*, the structural gene for ribosomal protein L10, can be cloned on a promoter-cloning vehicle, pGA24 (2). This cloning vehicle lacks the promoter for the tetracycline resistance genes (*tet*) but the *tet* structural genes are still intact. A *Hin*dIII site located adjacent to the structural genes for *tet* was used to clone a 2,164-bp *Hin*dIII fragment from λ21-10 (2, 18) by screening for insertional activation of *tet*. This 2,164-bp *Hin*dIII fragment was derived from a construction which involved exchanging the natural order of two *Eco*RI-*Hin*dIII fragments; one of these is a 1,874-bp fragment carrying most of the *rplKA* operon (except a small portion of the 5'-distal end of *rplK*) and its promoter region, plus the intercistronic region between *rplA* and *rplJ*, as well as most of *rplJ* except its 3'-distal region. The other *Eco*RI-*Hin*dIII fragment is a 290-bp fragment carrying the 3'-distal region of *rplJ* and the 5'-portion of *rplL* (see Fig. 2A). In the resulting plasmid, pGA26, the *tet* structural genes were shown to be under the control of the bacterial promoter, which we call *rplJp* (according to the notation suggested by Bachmann and Low [4]), located on the 2,164-bp *Hin*dIII fragment, since a strain carrying pGA26 became resistant to tetracycline at 20 μg/ml whereas a strain carrying pGA24 is sensitive at 1 μg/ml.

To determine that *tet* is transcribed from *rplJp*, six in vitro deletions were made from pGA26 by partial *Pst*I digestion followed by ligation. The structures of these deletion plasmids are shown in Fig. 2A. Inspection of the structure of these plasmids and the level of tetracycline resistance which they endow reveals that only when the 922-bp *Pst*I fragment known to carry *rplJp* (33) is present is there any measurable tetracycline resistance. This was corroborated by construction of pGA63 (Fig. 2B); this plasmid carries the 922-bp *Pst*I fragment derived directly from λ d*rif*^{d}18 and pGA46 (2). A strain carrying pGA63 became resistant to tetracycline at 20 μg/ml, which is the same as the level observed for the strain carrying pGA26. We conclude that the expression of *tet* from plasmid pGA26 and its derivatives must be initiated from *rplJp* and that there is no additional promoter in the region examined.

The expression of ribosomal protein structural genes in the maxicell strain CSR603 (39) transformed with various plasmids has been determined. The maxicells were irradiated with UV light and then were labeled with [^{35}S]methionine, as described in Materials and Methods. The labeled proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 3). In pGA26 only one additional protein, whose molecular weight is 16,000 (16K), was synthesized compared with the vector (pGA24) alone. The synthesis of this protein was observed when plasmids pGA34, pGA36, and pGA37 were in the maxicell strain. However, expression of this protein was not detected when plasmids pGA33, pGA35, and pGA39 were present in the maxicell strain. Therefore, the 16K protein must be a product of a truncated *rplJ* gene whose C-terminal end (19 amino acids) is deleted. Since *rplJ* was expressed whenever *tet* was activated (see Fig. 2) these two genes must be cotranscribed from a common promoter, *rplJp*. The expression of L1 was not observed in any of the plasmids tested, even though the L1 structural gene, *rplA*, is intact. These results support the above observation that *rplJp* is the only promoter located in the 2,164-bp *Hin*dIII fragment.

The intact operon, including the ribosomal protein genes, *rplJL*, and the RNA polymerase subunit genes, *rpoBC*, was analyzed in a second series of experiments. There are several lines of evidence indicating that these genes are cotranscribed (28, 45). However, there is a possibility that another promoter sequence is located in this region (14, 17). A plasmid carrying all four genes of the operon, *rplJ*, *rplL*, *rpoB*, and *rpoC*, was constructed by inserting in the unique *Hin*dIII site of pGA36 (see Fig. 2) the 9.5-kbp *Hin*dIII fragment from pNF1310 (14) that carries part of *rplJ*, all of *rplL*, and all of *rpoBC*. Since the *rpoB* gene was originally derived from

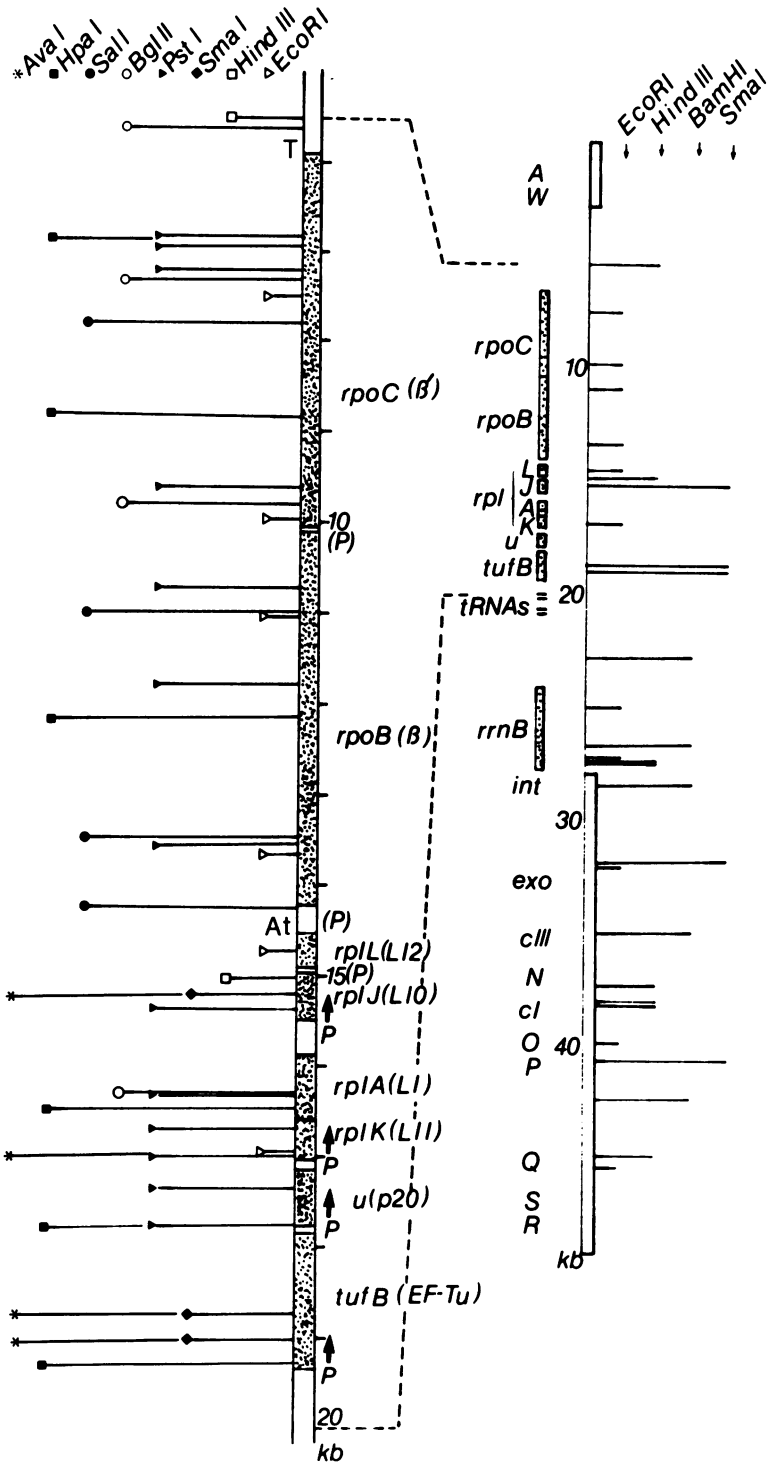

FIG. 1. *Restriction endonuclease map of λ drif^{d}18. The right-hand section of the figure illustrates restriction endonuclease sites on λ drif^{d}18, along with genes carried on the phage. The double lines represent λ DNA and the single line indicates bacterial DNA. A fine restriction endonuclease map of the bacterial DNA region from tufB to rpoC is expanded in the left-hand section of the figure. Location of the four ribosomal protein genes was obtained from the known DNA sequence (33). Approximate positions of rpoB, rpoC, tufB, and U are also shown (14, 46). P, Major promoters; (P), secondary promoters; At, attenuator; and T, transcription termination site.*

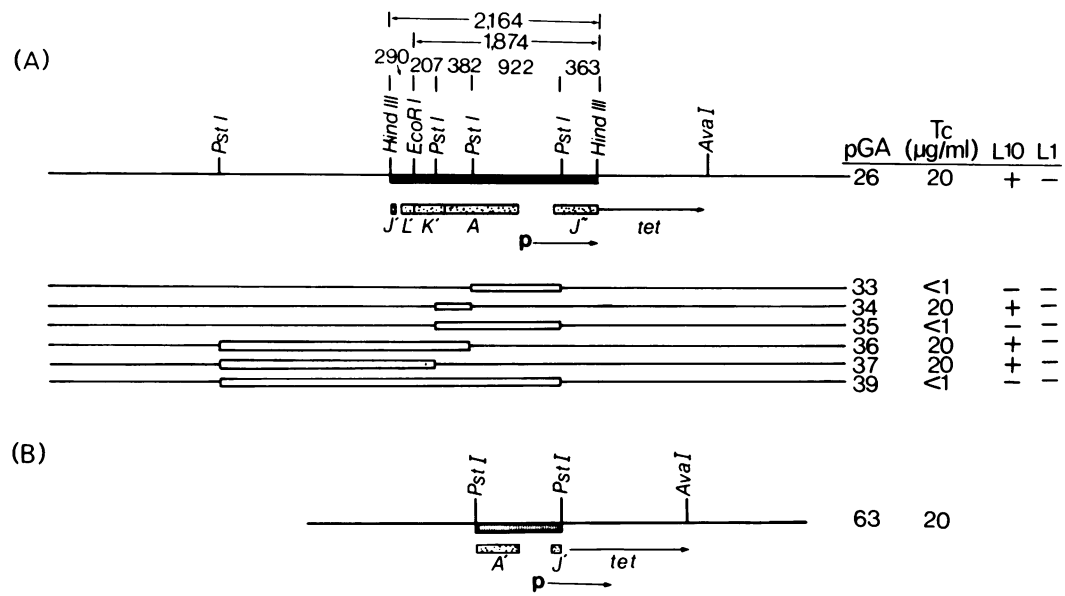

FIG. 2. *(A) Structure of pGA26 and its derivatives. (B) Structure of pGA63. The single line on pGA26 indicates plasmid pGA24, which was used as the vehicle for cloning the 2,164-bp HindIII fragment (the striped box) in the construction of pGA26. The single line on pGA63 indicates plasmid pGA46, which was used as the vehicle for cloning the 922-bp PstI fragment (the striped box). The locations of ribosomal protein genes are indicated by the stippled boxes. The following abbreviations are used: J' and J" (portions of rplJ), L' (a portion of rplL), K' (a portion of rplK), A (rplA), A' (a portion of rplA). P refers to the promoter, rplJp, for rplJL-rpoBC. The six deletion plasmids, derived from pGA26 by partial restriction endonuclease PstI digestion, are also shown; the deletions are represented by the open boxes. All plasmids are shown in linear form derived by opening at the EcoRI site in the chloramphenicol resistance gene. The length of the restriction fragments is indicated in base pairs. The tetracycline resistance level of cells carrying the different plasmids is shown on the extreme right-hand side of the figure. The expression of L1 and a portion of L10 is indicated by + for expression or − for lack of expression.*

λ *drif*^{d}18, which contains a dominant mutation in *rpoB* conferring resistance to the antibiotic rifampin (20), a strain that carries this recombinant plasmid, which was designated pGA43 (Fig. 4), became resistant to rifampin. However, this strain was sensitive to tetracycline at 1 μg/ml, even though the plasmid carries an intact *tet* structural gene (Fig. 4). In pGA43 the strong promoter, *rplJp*, initiates transcription toward *tet*; the fact that this plasmid did not bestow tetracycline resistance most likely indicates a region for transcription termination between *rpoC* and *tet*. This termination signal was removed from pGA43 by deletion of two *Bgl*II fragments that are located in *rplC*. As expected, cells carrying the resulting plasmid, pGA44 (Fig. 4), were resistant to tetracycline at 14 μg/ml. The *tet* genes expressed from pGA44 are most likely under the control of a promoter(s) located on the cloned bacterial DNA fragment.

The location of the promoter(s) on the bacterial portion of pGA44 was determined with the aid of seven deletion plasmids, pGA68 through pGA74, that were constructed by partial *Pst*I digestion of pGA44 followed by ligation. As with the previous set of plasmids analyzed (Fig. 2), inspection of these plasmids reveals that the major promoter for the operon lies in the 922-bp *Pst*I fragment (fragment A, Fig. 4). Plasmids pGA68, pGA69, and pGA73 all retain only the intercistronic region between *rpoB* and *rpoC*, yet the tetracycline resistance level was higher than in pGA43; this might be due to a weak promoter in the intercistronic sequence between *rpoB* and *rpoC* (5). Plasmid pGA78, in which the major promoter is deleted, still endows tetracycline (2 μg/ml) resistance. This is probably due to a minor promoter downstream from *rplJp* (see below).

The decreased amount of expression in pGA72 and pGA74 compared with that in pGA70 is quite likely due to the presence of an attenuator in the region between *rplJ* and *rpoB* (5, 11). To locate more precisely this attenuator, we have constructed two additional plasmids, pGA81 and pGA82, from pGA44 by partial *Eco*RI digestion and ligation (Fig. 4). In both plasmids, *rplJp* as well as *rplJ* and a part of *rplL* were retained.

These plasmids bestowed tetracycline resistance at 20 μg/ml, which is the same as for pGA70 and pGA71. Therefore, the position of the attenuator is most likely distal to *rplL*.

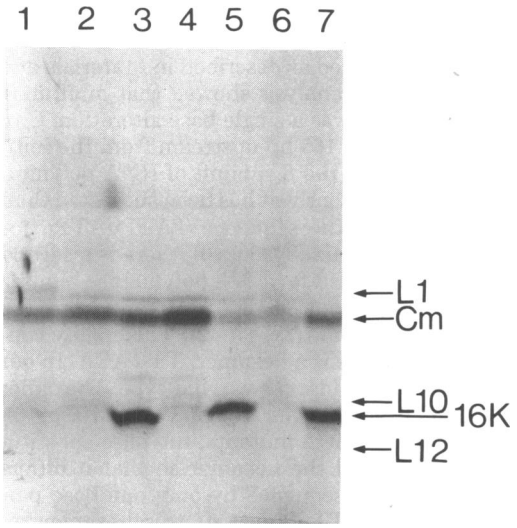

FIG. 3. *Polypeptides synthesized by plasmids in the maxicell strain. [^{35}S]methionine-labeled proteins were separated by 15% sodium dodecyl sulfate-polyacrylamide gel at 70 V. Lanes: (1) pGA24; (2) pGA39; (3) pGA26; (4) pGA33; (5) pGA34; (6) pGA35; (7) pGA36. The protein markers used are chloramphenicol acetylase (Cm) and ribosomal proteins L1, L10, and L12. The 16K protein refers to the product of truncated rplJ.*

To search for an additional minor promoter in the *rplJ-rplL* intercistronic region, a deletion plasmid, pGA128, was constructed from pGA78 by removal of two *Eco*RI fragments. pGA128 carries a *Pst*I-*Eco*RI fragment that contains the intercistronic region between *rpoJ* and *rplL* (Fig. 4). All other intercistronic regions have been removed from pGA128. It was observed that *tet* was still expressed at a low level from pGA128. Thus, we conclude that there might be a weak promoter in the *rpoJ-rplL* intercistronic region. A similar conclusion has been reached by Barry et al. (5).

As described above, pGA43, which contains intact *rpoBC*, has a region for transcription termination. This termination sequence must lie beyond *rpoC* because deletion of the two *Bgl*II fragments that lie in *rpoC* removed the terminator region (see above). To define this region, a plasmid, pGA55, was constructed in which most of the *rpoBC* genes were deleted from pGA43 by *Kpn*I digestion followed by ligation. There are five *Kpn*I sites in the *rplJL-rpoBC* operon; one is in a very early region of *rpoB* and the others are located within *rpoC*, near its 3'-end (see Fig. 1). Expression of *tet* was observed to be almost zero in pGA55 (Fig. 4). Therefore, the terminator region lies between the rightmost *Kpn*I site in *rpoC* and the *Bgl*II site that lies just outside of *rpoC*.

Analysis of an "up" mutation in an internal promoter between *rplL* and *rpoC*. Plasmid pGA78 (Fig. 4) bestows a very low level of

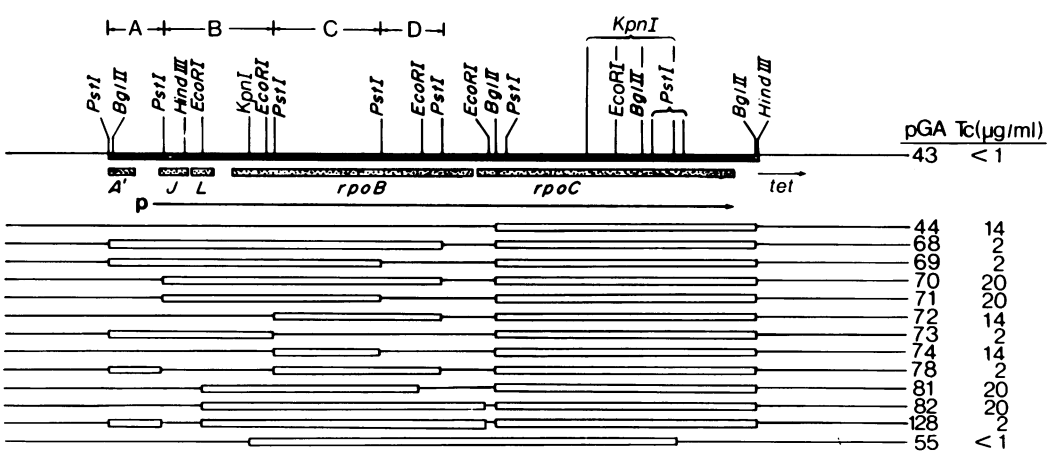

FIG. 4. *Structure of pGA43 and its derivatives. The single line on pGA43 indicates the DNA of the cloning vehicle, pGA24. Bacterial DNA is indicated by the striped box on pGA43. The locations of genes for ribosomal proteins and RNA polymerase subunits are indicated by the stippled boxes below pGA43. In the lower portion of the diagram the deletions in various plasmid derivatives of pGA43 are represented by open boxes. All plasmids are shown in linear form derived by opening at the EcoRI site in the chloramphenicol resistance gene. Tetracycline resistance levels of cells carrying the various plasmids are shown in the right-hand section of the figure.*

tetracycline resistance, which was nevertheless higher than basal level, indicating that there might be a weak promoter somewhere downstream from *rplJp*.

Mutations in this weak promoter were isolated by transforming mutagenized pGA78 into *E. coli* strain JF1161, selecting for chloramphenicol resistance, and screening for elevated tetracycline resistance. Most of the plasmid-carrying cells were sensitive to tetracycline above 2 μg/ml; however, about 1 in 10,000 colonies was resistant to a high level (12 to 24 μg/ml) of tetracycline. Sixteen independent tetracycline-resistant colonies were picked for further analysis. Plasmids were prepared from each of the 16 colonies as described in Materials and Methods, and these were transformed into strain JF1161. It was observed that all transformants that carried the mutant plasmids became resistant to the same concentration of tetracycline as did the original isolate. Therefore, the mutations must have occurred on the plasmid.

To localize the mutations, the mutant plasmids were digested with restriction endonuclease *Eco*RI, *Hin*dIII, or *Pst*I and were ligated; the ligated DNA mixture was used to transform strain JF1161. Chloramphenicol-resistant colonies were picked and tetracycline resistance was tested. In all cases more than 90% of the colonies became sensitive to the drug. Two of each class of tetracycline-sensitive colonies and tetracycline-resistant colonies were picked for restriction endonuclease analysis of the structure of the plasmids that they carried. It was found that all tetracycline-resistant cells carried the original mutant plasmid, whereas all sensitive cells harbored one of the shortened plasmids whose structure is shown in Fig. 5. In Fig. 5 are shown the structures of one mutant plasmid, pGA78-8, and its three classes of derivatives, pGA128-8, pGA39-8, and pGA68-8. The other 15 mutant plasmids were analyzed similarly. The results indicated that in all of the mutant plasmids the mutations were located between the *Eco*RI site in *rplL* and the *Pst*I site in *rpoB*. Since this region included the *rplL-rpoB* intercistronic region, it was concluded that the mutations were most likely in this region.

The DNA sequence of nine of these mutants has been analyzed as described in Materials and Methods. This analysis showed that in all nine mutants there was a single base alteration, C to T, at a position 165 bp upstream from the initiation codon for the β subunit of RNA polymerase (Fig. 6). Recently, it has been suggested that, in addition to the sequence TATPuATPu for transcription start (34, 35, 40), there is a second sequence required for RNA polymerase binding in a region about 35 bp upstream from the mRNA start point (37, 41, 42). This highly conserved sequence is a hexamer, TTGACA. In our mutant plasmids the wild-type hexamer G*C*GACA is changed to G*T*GACA (Fig. 6). Therefore, in these mutants, the base substitution has altered the hexamer so that it differs from the ideal sequence by only one base pair (GTGACA or TTGACA). If this hexamer sequence is really a part of the promoter, there should be a Pribnow sequence (TATPuATPu) about 15 to 20 bp downstream from it (37). In fact, there are two possible Pribnow sequences (Fig. 6). The sequence GTAAATC 15 bp downstream from the hexamer is similar to the known Pribnow sequence except for the first two base pairs. It should be noted that the sequence GT in the first two bases of the Pribnow sequence is not necessarily common. However, about 10% of known sequences from various promoters show variation at the first two bases (37). Another possible Pribnow sequence is TGAAATG, 26 bp downstream from the hexamer. Even though this sequence is very close to the prototype sequence of Pribnow, it might be too distant from the hexamer. Another common feature of

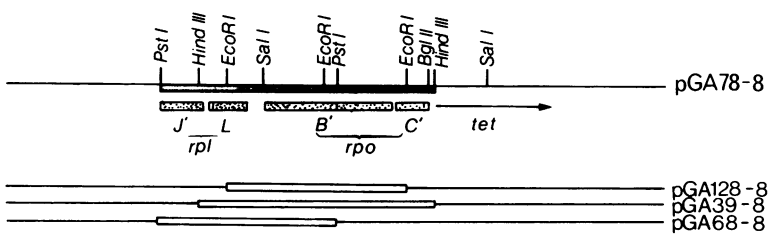

FIG. 5. *Structure of pGA78-8 and its derivatives. Bacterial DNA from the rif region is indicated by striped boxes on pGA78-8, which is shown in linear form derived by opening at the EcoRI site in the chloramphenicol resistance gene. The locations of ribosomal protein genes (J', a portion of rplJ; L, a portion of rplL) and RNA polymerase subunit genes (B', portions of rpoB; C', a portion of rpoC) are indicated by the stippled boxes. The structures of three deletion plasmids (pGA128-8, pGA39-8, and pGA68-8) constructed with restriction endonucleases EcoRI, HindIII, and PstI, respectively, are also illustrated. The deletions in these plasmids are represented by open boxes.*

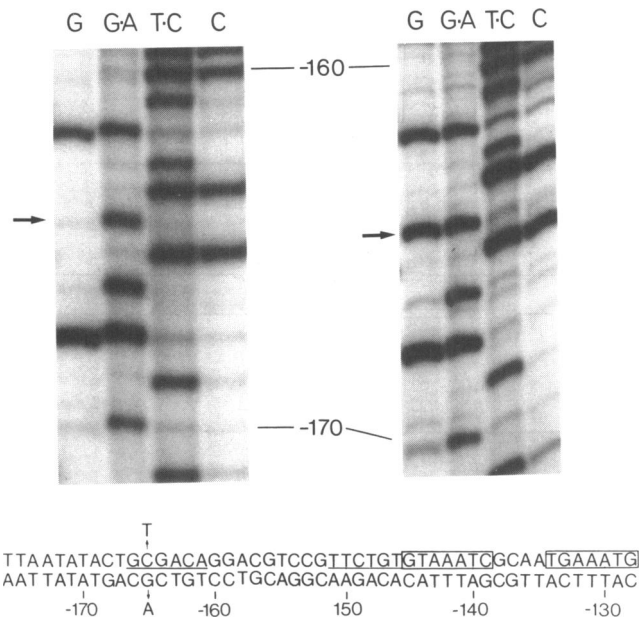

FIG. 6. *DNA sequence of the mutational alteration in pGA78-8. The 156-bp TaqI-SalI fragments from pGA78 (right) and mutant pGA78-8 (left) were purified, and the TaqI site was end-labeled with [α-^{32}P]dCTP, using DNA polymerase large fragment. DNA sequencing was according to Maxam and Gilbert (31). The numbers indicate base pairs preceding the ATG initiation codon for rpoB. The change, G to A, caused by the pGA78-8 mutation is indicated by arrows. Mutational alteration of the DNA sequence in pGA78 mutants and potential weak promoter sequences in the rplL-rpoB intercistronic region are shown in the lower section of the figure. Two potential Pribnow sequences are boxed. Two hexamers which are semiconserved in promoter binding sites are underlined. The numbers represent base pair preceding the ATG initiation codon for rpoB. The change, C/G to T/A, caused by a single mutation in the pGA78 mutants is indicated by an arrow.*

promoter sequences, that is, an adenine·thymine-rich region immediately preceding the hexamer TTGACA, was also found. The sequence TTCTGT adjacent to the first tentative Pribnow sequence is similar to the hexamer ATTTGT, which is also a relatively conserved sequence located adjacent to the Pribnow sequence (37).

These observations suggest that a weak promoter might be located in the *rplL-rpoB* intercistronic region in the wild type and that this promoter becomes stronger in our mutants because of a single base pair alteration.

Organization of the *rplKA* operon. A second operon for ribosomal protein structural genes, including *rplK* (L11) and *rplA* (L1) and lying adjacent to the *rplJL-rpoBC* operon, was examined by constructing a plasmid that carries these two ribosomal protein structural genes as well as the gene for protein U (14, 15) and the 3'-distal region of *tufB*, the structural gene for elongation factor EF-Tu (Fig. 7A). Plasmid pGA61 was constructed by cloning the 3.6-kbp *Sma*I fragment from λ d*rif*^{d}18 in the *Sma*I site on the promoter-cloning vehicle pGA39 (2) and screening for tetracycline resistance. The region containing *rpoJp* lies adjacent to the *tet* structural genes in pGA61 and was deleted by partial *Ava*I digestion of pGA61, followed by ligation. pGA66, the resulting plasmid (Fig. 7A), has *rplKp*, the promoter for L11, fused to *tet*. Strains carrying pGA66 were resistant to 32 μg of tetracycline per ml. To confirm this, another plasmid, pGA64, was constructed by cloning from λ d*rif*^{d}18 the 400-bp *Pst*I fragment that carries the *rplKp* region; it was observed that *rplKp* on this fragment strongly activated *tet* (Fig. 7B). A strain harboring plasmid pGA64 was resistant to tetracycline at 32 μg/ml, the same as the level observed in a strain carrying pGA66. We have already indicated that there is no promoter within the structural genes of *rplKA* (pGA33, Fig. 2). Therefore, we concluded that *rplKp* is the only promoter in the *rplKA* operon.

Promoter for gene U. The gene for the unknown function protein U lies immediately adjacent to *rplL* (46). To locate the promoter for this gene, a deletion plasmid, pGA90, was constructed from pGA61 by partial *Pst*I digestion and ligation (Fig. 7A). In pGA61 there are seven

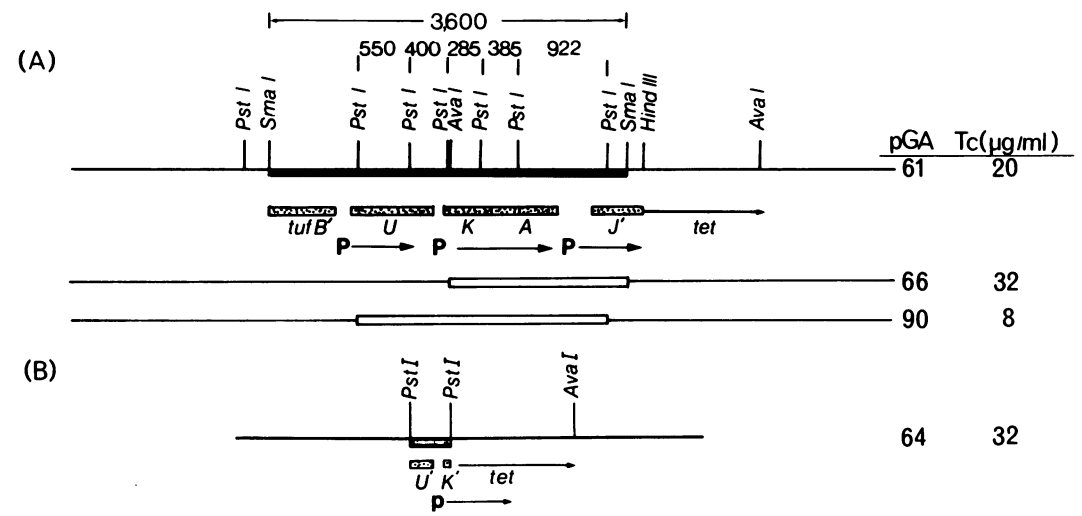

FIG. 7. *(A) Structure of pGA61 and its derivatives. (B) Structure of pGA64. The single line on pGA61 indicates pGA39, which was used as the cloning vehicle into which the 3.6-kbp SmaI fragment (the striped box) from λ drif*^{d}*18 (see Fig. 1) was inserted. The single line on pGA64 indicates pGA46, which was used as the vehicle for cloning the 400-bp PstI fragment (the striped box). The locations of each gene are indicated by the stippled boxes. Abbreviations used: tufB' (a portion of tufB), U (an unknown gene), U' (a portion of U), K (rplK), K' (a portion of rplK), A (rplA), J' (a portion of rplJ). P refers to promoters. pGA66 was derived from pGA61 by deleting the region extending from the AvaI site in rplK to the SmaI/AvaI site in rplJ. pGA90 was constructed from pGA61 by removing the five contiguous PstI fragments that carry the rplKA operon plus portions of rplJ and U. The deletions in pGA66 and pGA90 are represented by open boxes. The plasmids are shown in linear form derived by opening at the EcoRI site in the chloramphenicol resistance gene. The lengths of the SmaI fragment and the five PstI fragments are indicated in base pairs. Tetracycline resistance levels of cells carrying the various plasmids are shown in the right-hand section of the figure.*

*Pst*I sites, of which five are known exactly on the basis of DNA sequence (33). The position of the other two *Pst*I sites was deduced by digesting plasmid pGA61 with *Pst*I and analyzing the DNA fragment size. It was found that these two *Pst*I sites are, respectively, about 400 and 950 bp upstream from the *Pst*I site that is located in the early region of *rplK*. In pGA90 the five contiguous *Pst*I fragments that carry *rplJp* and *rplKp* were deleted (Fig. 7A), leaving the region containing the *tufB*-U intergenic region connected directly to *tet*. The expression of *tet* from this plasmid was examined by testing the tetracycline resistance level of a strain carrying pGA90; it was observed that *tet* was activated in pGA90. The promoter responsible for the expression of *tet* from this plasmid is probably the promoter for unknown protein U, since only U is located between the *rpoKA* operon and *tufB*, and the promoters for neither of these operons are present in pGA90. pGA90 bestowed resistance to tetracycline at 8 μg/ml. This value is lower than that observed with *rplJp* or *rplKp*, suggesting that the promoter for the gene coding for U might be weaker than either of the former.

Promoter for *tufB*. Another gene located close to the four ribosomal proteins described above is *tufB*, which is one of the two genes encoding elongation factor EF-Tu (19, 20). It has been proposed that *tufB* is transcribed from a promoter located downstream from the rRNA operon *rrnB* (27). However, it is not known whether *tufB* is cotranscribed with other genes that might be located between *tufB* and the rRNA operon *rrnB*, such as four tRNA genes (38). To extend our knowledge of the genetic organization of this region, we have constructed plasmids that carry *tufB* in the promoter-cloning vehicle pGA46. The 3.9-kbp *Pst*I fragment carrying the *tufB* gene derived from λ d*rif*^{d}18 was inserted in both orientations into a unique *Pst*I site located adjacent to *tet* in pGA46 (Fig. 8). In pGA100, in which the transcription of *tufB* and U is toward *tet*, the *tet* gene was expressed because it is now under the control of the promoter for U, whereas in pGA101, in which the orientation is opposite, *tet* was not expressed. To measure the expression of the cloned *tufB* gene, minicell-producing strain RH17 was transformed with either pGA100 or pGA101, and minicells were purified. The minicells were labeled with [^{35}S]methionine and the radioactively labeled proteins were electrophoresed in 10% sodium dodecyl sulfate-polyacrylamide gels (Fig.

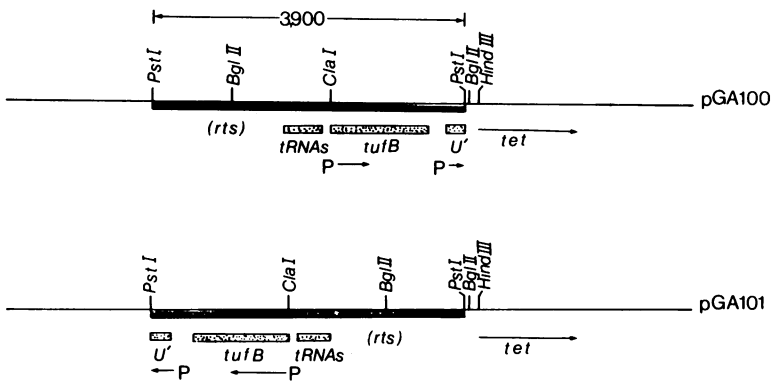

FIG. 8. *Structure of pGA100 and pGA101. The single line on pGA100 and pGA101 indicates pGA46, which was the cloning vehicle into which was inserted the 3.9-kbp PstI fragment (the striped box) from λ drif^{d}18 containing a portion of gene U, tufB, four tRNA genes, and rts. The location of the tufB, four tRNA genes, and rts have been determined by nucleotide sequencing data (An and Friesen, in preparation). P refers to promoters. The plasmids are shown in linear form derived by opening at the EcoRI site in the chloramphenicol resistance gene.*

9). Autoradiography of the gel indicated that both of these plasmids equally expressed the *tufB* function. Therefore, the promoter for *tufB* is probably located on the 3.9-kbp *PstI* fragment. We have recently found that the gene *rts* (15) is located very close to, but not in, *tufB* (unpublished data). The *rts* gene is also located on pGA100 and pGA101. Nucleotide sequencing data (unpublished data) indicate clearly that *rts* and *tufB* are not cotranscribed. Indeed, these same data show that four tRNA structural genes (38) are located between *rts* and *tufB* and that all five genes might be cotranscribed.

DISCUSSION

This work has provided an example of how one can study the organization of the transcriptional unit of bacterial chromosomal genes by using promoter-cloning vehicles developed recently (2). The observed results with such a system are in excellent agreement with previous results and also extend our knowledge of the region of 88 min on the *E. coli* genetic map. This method should be generally applicable to other regions of the bacterial genome. We have also shown that this system can be used to detect a mutationally altered promoter.

We have observed only one major promoter, *rplJp*, for the *rplJL-rpoBC* operon. Previously, Yamamoto and Nomura (45) and Linn and Scaife (28) independently reported that *rpoBC* genes are cotranscribed with *rplJL*. The expression of *rpoBC* genes was observed only when the region carrying *rplJp* was present. However, Fiil et al. (14) observed the noncoordinate expression of *rpoBC* and *rplL* with *rplJ* in two plasmids (pNF1492 and pNF1493). These plasmids expressed *rpoBC* and *rplL* but not *rplJ*. This observation suggested the possibility that there might be separate promoters for *rplL* and *rpoBC*, on the one hand, and *rplJ*, on the other. This idea was supported by the observation that L12 and RNA polymerase subunit β were efficiently synthesized in vitro from a DNA fragment that lacks the *rplJp* region and the 5'-end of *rplJ* (17). RNA polymerase-binding experiments also indicated that there might be an additional promoter site for *rplL* within the structural gene for L10 (10, 17). However, by using more sensitive techniques that allow RNA polymerase to bind and initiate on specific DNA restriction fragments, Taylor and Burgess (43) failed to find a polymerase binding site in the region. The present work strongly suggests that there is only one strong promoter in this operon. However, several derivatives of pGA43 revealed the presence of secondary promoters that are located in intercistronic regions of the *rplJL-rpoBC* operon. Previously (16), we failed to observe the presence of weak promoters in these regions (16). However, when a longer incubation period on tetracycline agar was adopted, we were able to observe a clear and reproducible difference between plasmids carrying weak promoters and those carrying no promoters (see also Materials and Methods). Weak promoters were also observed by Barry et al. (5). Experiments by Holowachuk et al. (18) also revealed a weak promoter located between either *rplJ* and *rplL* or *rplL* and *rpoB*.

It was suggested that *rplKA* is a separate operon having its own promoter. We have observed that the promoter for the *rplKA* operon lies in a 400-bp *PstI* fragment that contains the

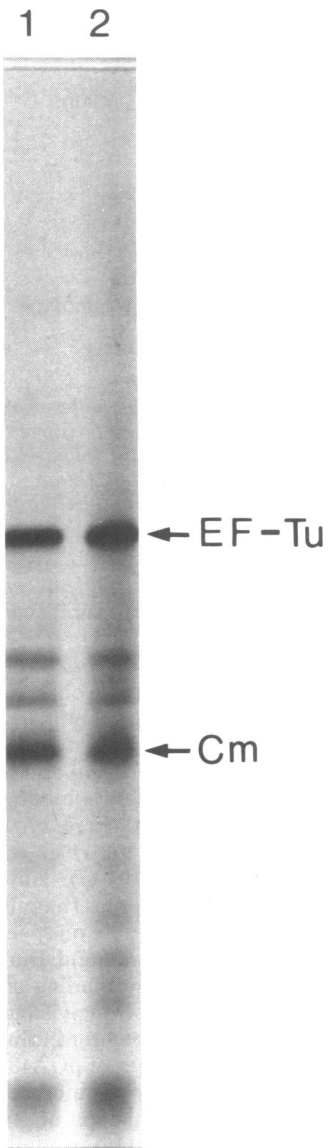

FIG. 9. *Autoradiogram of labeled polypeptides synthesized by plasmids in the minicell-producing strain. [^{35}S]methionine-labeled proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel at 70 V. Lanes: (1) pGA100; (2) pGA101. The arrows indicate EF-Tu and chloramphenicol acetylase (Cm).*

intercistronic region between *rplK* and U. Post et al. (33) have published the DNA sequence of a part of this 400-bp *Pst*I fragment and suggested that the region near the sequence TACAATT 100 bp upstream from the initiation codon of L11 might be a promoter sequence for the *rplKA* operon.

We have also found an independent promoter for U. A similar result was obtained by Yamamoto and Nomura (46). The presence of an independent promoter for *tufB* was suggested by Linn et al. (27). They have isolated several independent phages that carry a fusion of *argCBH* to *tufB* and have demonstrated that *rplK* and *rplA* do not share a promoter with *tufB*. In such fusions, the expression of *tufB* was observed only when *arg* was derepressed, whereas the expression of the genes for protein U and four ribosomal proteins were independent of the repression. However, Linn et al. (27) were unable to localize the position of the *tufB* promoter. In the present work, we have localized the possible position of this promoter to a region between *tufB* and the *Pst*I site that lies about 1.2 kbp upstream from the *tufB* gene. From other work (G. An and J. D. Friesen, manuscript in preparation), we now know that *tufB* is not cotranscribed with *rts* (15), a gene that is located near *tufB*.

Our results indicate that the relative amount of *tet* expression might be dependent on the strength of promoters cloned into the promoter-cloning vehicles. For example, *rplKp* bestowed tetracycline resistance to 32 μg/ml, *rplJp* bestowed resistance to 20 μg/ml, and the gene U promoter bestowed resistance to 8 μg/ml. This is in agreement with earlier experiments, which showed that the expression of the gene U in UV-irradiated cells infected with λ d*rif*^{d}18 is lower than that of the *rplKA* operon or the *rplJL-rpoBC* operon (46). However, although it is clear that there is a relationship between the level of *tet* gene expression and the drug resistance level of cells, this relationship is not necessarily linear. Attempts to measure directly by gel electrophoresis the level of *tet* gene product expression in cells carrying various plasmids have not been successful (unpublished data). Nevertheless, the level of cellular drug resistance is a function of the strength of *tet* gene expression, which in turn in our system depends on the strength of the promoter that is driving *tet*. The system is clearly usable in a semiquantitative manner. This is demonstrated by the fact that we have been able to isolate several promoter "up" mutations by using one of our plasmids. Analysis of the DNA sequence of nine of these mutations showed that there is a single base alteration, C to T, at a position 165 bp upstream from the initiation codon for the β subunit of RNA polymerase. The base substitution in these occurred at the DNA sequence of the −35-bp region in the RNA polymerase binding site.

There are a number of known promoter mutations that show alteration at the −35-bp region (37). Our experiment provides additional evidence that the sequence at the −35-bp region

from the mRNA start point is important for RNA polymerase function. These observations suggest that a weak promoter might be located downstream from the *rpoB* structural gene and that this promoter becomes stronger by mutation in the RNA polymerase binding site. The biological significance of the presence of the weak promoter in this region is not known. It has been observed that the transcription of *rpoBC* is not coordinated with *rplJL* (6, 12, 13, 29, 36); thus, it is possible that the weak promoter we have found in the region preceding *rpoB* might be involved in this noncoordinate regulation.

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