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Regulation of ribosomal RNA promoters with a synthetic *lac* operator

(RNA quantitation/stable 4.5S RNA/chloramphenicol acetyltransferase/21-base-pair *lac* operator/*rrnB* promoters P_1 and P_2)

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ABSTRACT **A synthetic 21-base-pair long DNA fragment containing the central *lac* operator sequence has been inserted near the initiation point of the cloned *Escherichia coli rrnB* rRNA promoter P_2 in the natural and reverse orientation. RNA synthesis is efficiently repressed in both orientations in *lac* I^q strains and is induced with isopropyl β-D-thiogalactoside. When the *rrnB* promoter P_1 is also present, upstream from P_2 and the synthetic *lac* operator, repression of transcription is incomplete. The levels of transcription were measured *in vivo*, indirectly by the expression of a protein (chloramphenicol acetyltransferase), or directly by the expression of a stable RNA (*E. coli* 4.5S RNA) in a simple assay involving gel electrophoresis of unlabeled total RNA from *E. coli*. The *rrnB* promoter constructions can produce high levels of protein expression as well as high levels of expression of stable RNA.**

Since many gene products are detrimental to the cell if overproduced (e.g., see ref. 1), expression systems demand an inducible strong promoter. Such a promoter can be generated by two approaches: (*i*) an inducible but weak promoter is modified to strengthen the promoter, or (*ii*) a strong constitutive promoter is made inducible by the addition of a regulatory element.

A number of mutants for the *lac* promoter (2–6) are known. The most prominent, a double mutation in the Pribnow box, results in the stronger *lacUV5* promoter (7). More recently, the strength of this promoter has been further increased by at least a factor of 5: the −35 region of the *lacUV5* promoter was replaced with the corresponding sequences from the *trp* promoter, yielding the *tac* promoter (8–10), which still can be regulated in the same manner as the *lacUV5* promoter.

The second means of constructing an expression system was recently applied to the promoter for the *Escherichia coli* outer membrane lipoprotein (*lpp*) gene. This promoter was made inducible by downstream placement of the *lacUV5* promoter and operator (11, 12). However, in the system tested, this manipulation renders the *lpp* promoter less efficient by a factor of at least 3 (11, 12).

The *rrnB* promoters are tandem promoters (P_1 and P_2) ≈118 nucleotides apart. In a separate study, we have shown by nuclease S1 mapping (13, 14) that both P_1 and P_2 are active *in vivo* when located on the 185-base-pair (bp) *Fnu*DII fragment from pKK3535 (positions 1162–1346; see refs. 15 and 16), which contains little flanking sequence (unpublished results). Up to now, we had only indirect evidence that the rRNA promoters contribute to the expression of proteins when fused to the respective genes (1). Here, we show directly that the rRNA promoters can produce high levels of the protein chloramphenicol acetyltransferase.

We describe the regulation of the *rrnB* rRNA promoters using only a synthetic *lac* operator of 21 bp (a generous gift of K. Itakura and P. Dembeck) without significant loss of promoter activity. When we insert the *lac* operator in either orientation near the transcription start, P_2 is regulated. However, if P_1 is placed upstream from P_2 and the operator, the combined promoters have a residual transcriptional activity of 30%–50% under repressed conditions.

A strategy for evaluating promoter activity *in vivo* is introduced. We directly measure a stable RNA, the 4.5S RNA from *E. coli*, whose gene (a generous gift of M. Fournier; see ref. 17) is positioned downstream from the promoter to be assayed. Thus, we avoid the comparison of translations of different hybrid mRNAs, which can lead to unrealistic pictures of the transcriptional activity of different promoters.

MATERIALS AND METHODS

Strains, DNA. *E. coli* strain RB791 (W 3110 I^q, L8; see ref. 18) was obtained from Roger Brent. DH20 (DH1 carrying F′ *lac* I^q) and DH21 (DH1 carrying F′ *lac* I^{sq}) were obtained from Doug Hanahan (19).

Bacteria were grown at 37°C in YT medium (20) supplemented with the appropriate antibiotic. Preparation of DNA, fill-in reactions, ligations, and transformations were carried out as described (21, 22). Two complementary oligonucleotides of 21 nucleotides containing the *lac* operator (5′ A-A-T-T-G-T-G-A-G-C-G-G-A-T-A-A-C-A-A-T-T and 5′ A-A-T-T-G-T-T-A-T-C-C-G-C-T-C-A-C-A-A-T-T) were a gift of K. Itakura and P. Dembeck. The 223-bp *Hha* I fragment containing the 4.5S RNA gene (17) was donated by M. Fournier. Small amounts of plasmid DNA for restriction mapping were prepared by the alkali method (23). All plasmid constructions were verified by restriction mapping with several enzymes and the relevant portions of pKK176-2, pKK176-3, pAH3-4, pAH4-1, pAH9-2, pAH10-2, pKK267-1, pKK268-1, pKK279-1, pKK287-12, pKK289-2, and pKK299-3 were verified by DNA sequencing (24).

Protein Gels. Twelve percent acrylamide protein gels for the quantification of chloramphenicol acetyltransferase were prepared according to ref. 25 and stained with Coomassie blue. The gels were photographed and scanned on a Beckman DU-8 spectrophotometer/gel scanner.

Nucleic Acid Isolation. Freshly transformed cells were grown to a density of A_{550} = 0.65. Aliquots of each culture (2 × 1.4 ml) were spun for 20 sec in an Eppendorf centrifuge at 4°C. The pellet was kept at −80°C if not processed immediately. For plasmid DNA isolation (23) and quantitation, one aliquot of the cell pellet was used. The DNA was dissolved in 20 μl of H_2O. Ten microliters was electrophoresed on 1% agarose gels in electrophoresis buffer [50 mM Tris acetate (pH 8)/20 mM sodium acetate/1 mM EDTA]. The gels were stained in ethidium bromide and recorded with Polaroid 665 film, which also provides a negative. The negative was used

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Abbreviations: IPTG, isopropyl β-D-thiogalactoside; bp, base pair(s).

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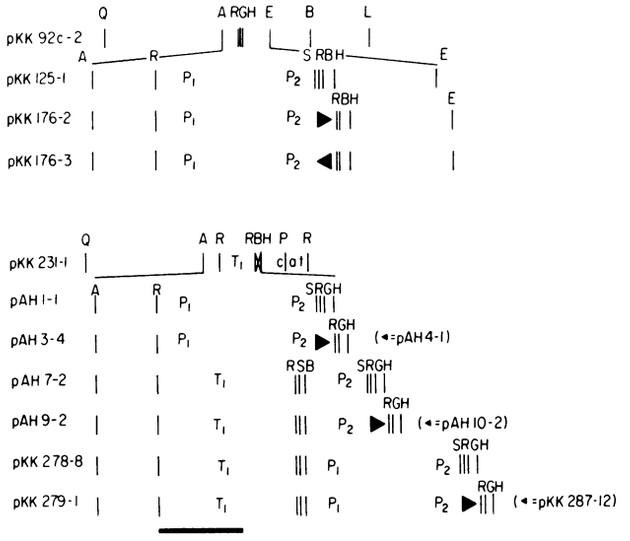

FIG. 1. Construction of rRNA promoters containing a synthetic *lac* operator. The 185-bp *Fnu*DII fragment from pKK3535 (positions 1162–1346; see refs. 15 and 16) has been ligated to *Eco*RI linkers (G-G-A-A-T-T-C-C) and cloned into the *Eco*RI site of promoter probe vector pKK92c-2 (28) yielding pKK125-1, which confers resistance to tetracycline. Addition of the *Eco*RI linkers generates a unique *Sma* I site just downstream from *rrnB* promoter P_2. The two synthetic nonphosphorylated (to avoid multiple insertions) strands containing the *lac* operator have been annealed and blunt-end ligated to pKK125-1 linearized with *Sma* I. Plasmids with inserts were identified by the increased size of the *Eco*RI fragment containing the *rrnB* promoters P_1 and P_2. Two candidates, pKK176-2 (which is actually a plasmid dimer in which only one *Sma* I site contains an insert) and pKK176-3 were sequenced around the site of insertion (24). The sequence of pKK176-2 (starting with the overlined Pribnow box of promoter P_2) is as follows: T-A-T-T-A-T-G-C-A-C-A-C-C-C-C-*A-A-T-T-G-T-G-A-G-C-G-G-A-T-A-A-C-A-A-T-T*-G-G-G-A-A-T-T-C. The *in vitro* transcription initiation points of the wild-type P_2 promoter (27) are marked by dots, the 21-bp *lac* operator sequence is shown in italics (which interrupts the *Sma* I site C-C-C-G-G-G) and the *Eco*RI site is underlined. *In vivo* transcription initiation (29) has been linked in *rrnA* (which is identical to the *rrnB* promoter) to one of the four cytosine residues and the following guanine residue (which is replaced here by the first adenine of the synthetic operator). In pKK176-3, the operator is in the reverse orientation. The *Pst* I/*Hin*dIII fragments of pKK125-1, pKK176-2, and pKK176-3 (containing part of the β-lactamase gene) were inserted into pKK231-1 (replacing the 950-bp *Pst* I/*Pst* I/*Hin*dIII segment) yielding pAH1-1, pAH3-4, and pAH4-1, respectively. Generally only one orientation of the operator in otherwise identical constructions is shown. Corresponding plasmids with the opposite orientation are denoted in parentheses. The operator in the natural orientation is marked by ▸ and in the reverse orientation by ◂. In pKK231-1 (28), part of the chloramphenicol acetyltransferase gene is marked "cat." To eliminate *rrnB* promoter P_1 upstream from P_2, the *Dde* I site between the promoters (position 1274; see ref. 16) in pAH1-1, pAH3-4, and pAH4-1 was filled in and *Bam*HI linkers (C-C-G-G-A-T-C-C-G-G) were added. The *Bam*HI/*Hin*dIII fragments containing P_2 were cloned between the *Bam*HI and *Hin*dIII sites (located in the polylinker) of pKK231-1 (28), yielding pAH7-2, pAH9-2, and pAH10-2, respectively. A further series of plasmids was made to place the constructions including P_1 (in analogy to pAH7-2, pAH9-2, and pAH10-2) downstream from *rrnB* transcription terminator T_1 of pKK231-1: the *Bam*HI/*Hin*fI fragment pKK34-121 (27), which has a *Bam*HI site upstream from *rrnB* promoter P_1: G-G-A-T-C-C-G-G-T-C-A-G-A [the *Bam*HI site is overlined; nucleotide 1162 of *rrnB* (16) is underlined]. This fragment containing promoter P_1 was ligated with the *Hin*fI/*Eco*RI fragments (containing P_2 with or without operator) of pAH1-1, pAH3-4, and pAH4-1, respectively, as well as the *Eco*RI/*Bgl* II/*Hin*dIII polylinker from pKK92c-2 (28) and the pKK231-1 *Bam*HI/*Hin*dIII backbone to yield plasmids pKK278-8, pKK279-1, and pKK287-12, respectively. Restriction sites are abbreviated as follows: A, *Aat* II; B, *Bam*HI; E, *Eco*RV; G, *Bgl* II; H, *Hin*dIII; L, *Sal* I; P, *Pvu* II; Q, *Pvu* I; R, *Eco*RI; S, *Sma* I; T, *Pst* I.

for scanning and estimation of the plasmid amount. For RNA isolation, a method slightly modified from ref. 26 was used. The cell pellet was resuspended in 500 μl of cold 50 mM Tris·HCl (pH 7.5)/5 mM $MgCl_2$. The material was twice extracted with the same volume of liquefied phenol (Fisher A-931). Forty microliters of 20% potassium acetate (pH 4.5) and 1 ml of ethanol was added, and the nucleic acid was precipitated. The pellet was washed in 95% ethanol, dried, and dissolved in 50 μl of double-distilled sterile H_2O. Seven microliters was electrophoresed on 12% acrylamide/0.4% bisacrylamide gels (130 × 180 × 1.5 mm; the well is 5 mm wide) in 90 mM Tris borate (pH 8.3)/2.5 mM EDTA at 300 V and 4°C until the xylene cyanol marker dye migrated ≈180 mm. The gel was stained in ethidium bromide and recorded as described for DNA gels.

RESULTS

Plasmid constructions were done as outlined in the figure legends. The 21-bp operator fragment has been cloned close (see Fig. 1 legend) to the transcription initiation site(s) (27, 29) of the ribosomal RNA promoter P_2 in both orientations.

The constructions with the *rrnB* tandem promoters on a small DNA fragment with and without *lac* operator were transferred to pKK231-1 (28) to monitor levels of chloramphenicol acetyltransferase expression. When strain RB791 (which is a *lac* repressor overproducer) was transformed with pAH1-1, pAH3-4, and pAH4-1 (Fig. 1) and plated on YT medium containing chloramphenicol (100 μg/ml), the colonies grew in the absence and presence of the inducer isopropyl β-D-thiogalactoside (IPTG). This indicates that either the 21-bp synthetic operator is not functional in that position downstream from P_2 or that the upstream promoter P_1 overrides the repressor to a certain degree, thus allowing transcription of the chloramphenicol acetyltransferase gene. To discriminate between these two possibilities, the P_1 promoter in the aforementioned vectors was eliminated to yield pAH7-2, pAH9-2, and pAH10-2, respectively (Fig. 1).

Uninduced, only pAH7-2, which lacks the operator, grew on YT plates containing chloramphenicol at 100 μg/ml. All three constructions grew on these plates in the presence of IPTG. This finding indicates that rRNA promoter P_2 can be regulated with the synthetic *lac* operator. Further confirmation was obtained by analyzing the total protein of strains harboring the various constructions. We can see that the rRNA promoters, if induced, contribute to the expression of high levels of chloramphenicol acetyltransferase (see Figs. 3 and 5) and that P_2 alone is efficiently regulated with the synthetic *lac* operator. Both orientations of the synthetic *lac* operator allow an almost complete repression of the P_2 promoter (when cells containing pAH9-2 or pAH10-2 are plated on medium containing <15 mg of chloramphenicol per ml or during prolonged incubation, small resistant colonies are observed). The pAH10-2 (P_2O_R; rRNA promoter with *lac* operator in reverse orientation) construction can be induced to similar chloramphenicol acetyltransferase levels as the pAH7-2 construction (P_2), while the pAH9-2 (P_2O_N; rRNA promoter with *lac* operator in natural orientation) construction has about one-half its activity (see Figs. 3 and 5). In constructions that include upstream promoter P_1, we do not observe such a pronounced difference in chloramphenicol

A mark that does not carry a sign for a restriction site has the same site as the mark precisely above it. In pKK125-1, pKK176-2, and pKK176-3, the relevant regions between the *Aat* II and *Eco*RV sites are shown enlarged. In pAH1-1 and constructions below, the relevant regions between the *Aat* II and *Hin*dIII sites are shown enlarged. The bar indicates 100 bp on the enlarged scale. The X in pKK231-1 denotes the *Eco*RI/*Sma* I/*Bam*HI/*Sal* I/*Pst* I/*Hin*dIII polylinker.

acetyltransferase levels with and without operator in either orientation.

To exclude the possibility that in pAH1-1, pAH3-4, and pAH4-1, clockwise-transcribing pBR322 promoters contribute to chloramphenicol acetyltransferase expression (and, therefore, prevent complete repression), the constructions analogous to pAH7-2, pAH9-2, and pAH10-2, in which a terminator prohibits such upstream-initiated transcriptions were tested (pKK279-1, pKK278-8, and pKK287-12; Fig. 1). The results were identical, supporting the role of P_1 in readthrough (see also ref. 30).

Although we are able to show with promoter–chloramphenicol acetyltransferase fusions that the *rrnB* promoters can be regulated with a synthetic operator, we are not confident that relative amounts of expressed protein correspond closely to the transcriptional rate of a given promoter. This problem arises whenever the mRNAs transcribed from different promoters are not completely identical.

The 5′ untranslated region of the hybrid chloramphenicol acetyltransferase message transcribed from the *rrnB* promoter(s) is altered by the insertion of the synthetic *lac* operator (whose sequences are cotranscribed) in either orientation. This addition might have an effect on the stability (see below) or translatability of the mRNA. Thus, the accumulation of chloramphenicol acetyltransferase may not precisely reflect the transcriptional activity of the *rrnB* promoter(s) with and without *lac* operator.

Therefore, we developed a simple *in vivo* system for the measurement of promoter strength independent of transcript translation. An RNA is expressed that is processed into a stable RNA species, the 4.5S RNA [whose mature form consists of 114 nucleotides (17, 31)], that can be easily extracted with the total *E. coli* small RNA. Finally, the 4.5S RNA can be separated from the other stable RNA species on polyacrylamide gels, stained, and measured with a densitometer.

pKK223-3 (Fig. 2 *Upper*), which is used as an expression vector because it contains a variety of restriction sites (from pUC8; see ref. 32) for the insertion of genes to be expressed at high levels between the regulatable *tac* promoter (8–10) and the *rrnB* transcription terminators (15, 16), was used for the introduction of the 4.5S gene (17) into the unique *Sma* I site (for details see Fig. 2 *Lower*, including legend). When the gene is inserted in the correct orientation (the *tac* promoter transcribes the 4.5S gene) plasmid pKK235b-15 is obtained (Fig. 2 *Lower*).

When RB781 was transformed with pKK235b-15 and grown in the presence of 2.5 mM IPTG (under induced conditions), a strong band comigrating with authentic purified 4.5S RNA (a gift from M. Fournier) was observed. This finding demonstrates that the hybrid precursor RNA is correctly processed. RNA extracted from the host strains alone or from strains harboring pKK223-3 showed only a very faint band of equal intensity in this region, originating from the cellular single copy gene (see Fig. 4, host lane).

The *tac* promoter residing on a *Bam*HI/*Eco*RI fragment of pKK235b-15 was exchanged with *Bam*HI/*Eco*RI fragments containing the *rrnB* promoter constructions from pAH7-2, pAH9-2, pAH10-2, pKK278-8, pKK279-1, and pKK287-12 to yield pKK266-1, pKK267-1, pKK268-1, pKK290-2, pKK289-2, and pKK299-3, respectively (Fig. 2 *Lower*). The constructions were grown in RB791, DH20, or DH21, under repressed and derepressed (2.5 mM IPTG) conditions to a cell density of $A_{550} = 0.65$. While the amount of plasmid was identical in all preparations (data not shown), the amount of 4.5S RNA varied (see Fig. 4). When identical constructions were compared under repressed and derepressed conditions, a difference in the level of 4.5S RNA was only found in constructions that include the *lac* operator (see Fig. 4). In some of the lanes, it is apparent that longer RNA products than 4.5S RNA are present. These are precursors to 4.5S RNA

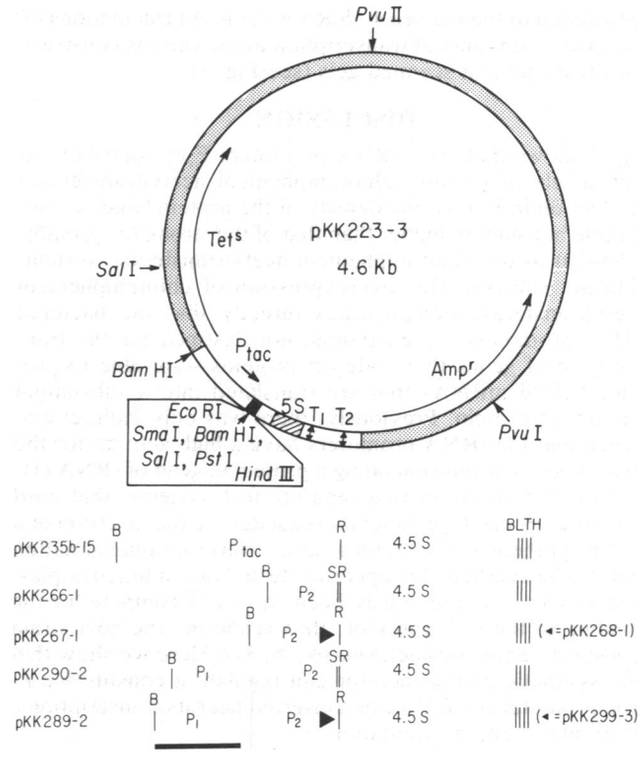

FIG. 2. Fusion of various promoters to the 4.5S RNA gene. (*Upper*) To construct pKK223-3, pKK10-2 (1) was digested with *Bam*HI and *Hin*dIII and the large fragment was isolated. ptac11 (10) was linearized with *Hin*dIII, filled in, and *Bam*HI linkers (C-C-G-G-A-T-C-C-G-G) were added followed by *Eco*RI digestion. The *Bam*HI/*Eco*RI fragment (≈260 bp) containing the *tac* promoter was ligated with the *Eco*RI/*Hin*dIII polylinker from pUC8 (32) to the pKK10-2 backbone. Finally, the *Pvu* I/*Bgl* I fragment containing part of the β-lactamase (*bla*) gene of pBR322 was exchanged against the analogous fragment from pUC8 (32). The latter plasmid has a point mutation in the *bla* gene that inactivates the *Pst* I site but maintains resistance to ampicillin (Amp^r). Thus, the resulting plasmid pKK223-3 contains a unique *Pst* I site in the polylinker region. Tet^s, tetracycline sensitive. (*Lower*) The 4.5S RNA gene lacking its promoter (provided as a 223-bp *Hha* I fragment by M. Fournier; see ref. 17) was blunt-ended and ligated into the *Sma* I site of pKK223-3, yielding pKK235b-15 with the insert in the correct orientation. The promoter-containing *Bam*HI/*Eco*RI fragments from pAH7-2, pAH9-2, pAH10-2, pKK278-8, pKK279-1, and pKK287-12 were ligated with the *Eco*RI/*Hin*dIII fragment containing the 4.5S gene from pKK235b-15 to the *Bam*HI/*Hin*dIII backbone from pKK10-2 (1), yielding pKK266-1, pKK267-2, pKK268-1, pKK290-2, pKK289-2, and pKK299-3, respectively. For abbreviations see legend to Fig. 1. Bar represents 100 bp.

because the same bands hybridize (in addition to the mature 4.5S RNA band) to a DNA probe (the 223-bp *Hha* I fragment) containing the 4.5S RNA gene during RNA blot analysis (not shown). On the same blot, no bands smaller than 4.5S are detected—a further indication of the stability of the RNA molecule. The precursor sizes were estimated by running the RNA on denaturing acrylamide gels containing 7 M urea (not shown). The P_1P_2 precursor migrated near the smaller band of human 7S RNA (ref. 33; a gift of R. Gilmore, Rockefeller University, New York), indicating a length of ≈270 nucleotides. The P_{tac} precursor comigrated with *E. coli* 6S RNA (184 nucleotides; see ref. 34) indicating a length of ≈180 nucleotides and the $P_2O_N/P_1P_2O_N$ precursor migrated somewhat faster than 6S RNA, indicating a size of ≈170 nucleotides ($P_1P_2O_N$ indicates tandem rRNA promoters with operator near P_2 in natural orientation). The amounts of these precursors (corrected for their respective sizes) have

been added to the mature 4.5S RNA values in calculations of the relative amounts of transcription in the various constructions from several scanned gels (see Fig. 5).

DISCUSSION

Fig. 3 shows that *rrnB* rRNA promoters P_1P_2 contribute to high levels of protein (chloramphenicol acetyltransferase) production in *E. coli* [the density of the protein band is comparable but slightly higher than that of the strong *tac* promoter (8–10) in the chloramphenicol acetyltransferase system; data not shown]. This overexpression of chloramphenicol acetyltransferase demonstrates directly that the bacterial rRNA promoters—even though not destined for the transcription of genes that code for proteins—are able to produce hybrid mRNAs that are translated into a substantial amount of protein. Previously, there was only indirect evidence that the rRNA promoters have a high activity for the expression of a gene encoding a protein instead of rRNA (1).

Figs. 3–5 show, in two separate test systems, that *rrnB* promoter P_2 can be efficiently regulated by the insertion of a 21-bp synthetic *lac* operator near its transcriptional initiation point. The synthetic *lac* operator, tested on a multicopy plasmid *in vivo*, has previously been shown to compete for the binding of the *lac* repressor, thus rendering the host strain constitutive for β-galactosidase (36, 37). Here we show that the synthetic 21-bp operator can regulate a constitutive *E. coli* promoter (*rrnB* P_2) when inserted near its transcriptional start site in either orientation.

Parallel constructions of the *rrnB* promoters fused to the chloramphenicol acetyltransferase and 4.5S RNA genes show levels of expression that are generally in agreement. However, the induced expression of chloramphenicol acetyltransferase in a construction containing P_2 and the *lac* operator in the natural orientation amounts to ≈50% that of P_2 alone (or P_2O_R), while only minimal differences appear between the same constructions in the 4.5S RNA system. This variation in the amount of protein could be due to an effect of the operator sequence (since it is cotranscribed) on the

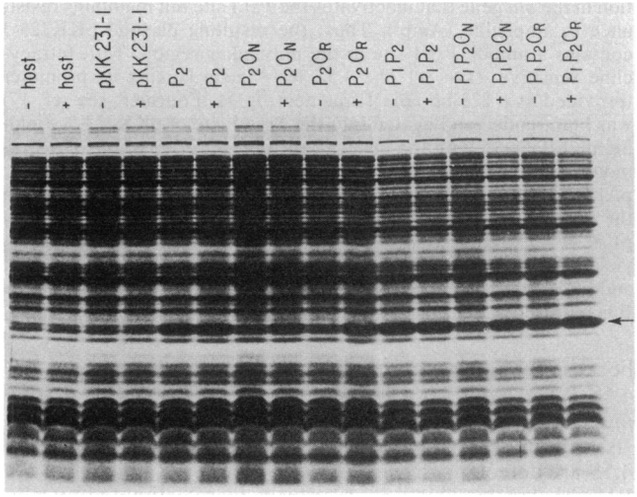

FIG. 3. Expression of chloramphenicol acetyltransferase with various promoter constructions. *E. coli* RB791 (18) containing the plasmids was grown in the absence (−) and presence (+) of 2.5 mM IPTG. At an approximate cell density of A_{550} = 1.0, 1 ml was harvested by centrifugation and dissolved in 50 μl of sample buffer (25). An aliquot of 10 μl was loaded on the gel. Arrow identifies the chloramphenicol acetyltransferase protein band, which comigrates with host proteins. Chloramphenicol acetyltransferase value estimates have been corrected for the contribution of the host bands. pKK231-1 (28) is the parent plasmid of the constructions tested and contains no promoter fused to the chloramphenicol acetyltransferase (*cat*) gene (see Fig. 1).

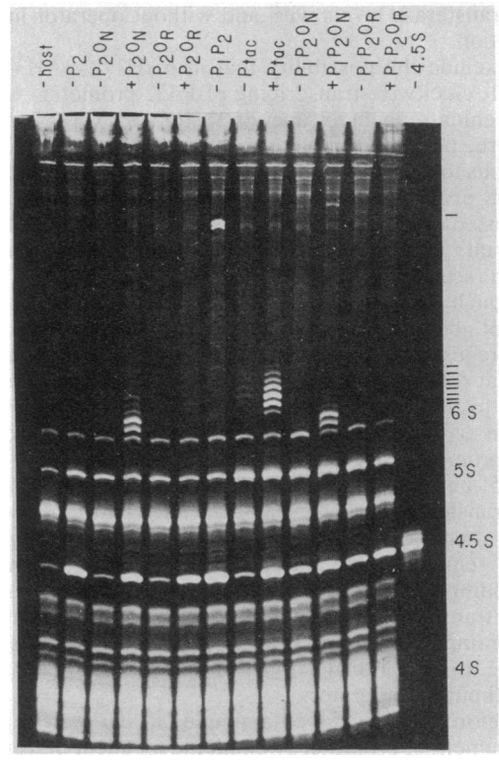

FIG. 4. Expression of the stable 4.5S RNA with various promoter constructions. Bacterial cultures (host strain DH20) were inoculated with a freshly transformed single colony and grown in the absence (−) or in the presence (+) of 2.5 mM IPTG. RNA was extracted and electrophoresed. The bands for 6S, 5S (there are random variations between the two forms of 5S RNA), 4S (tRNAs), and 4.5S RNA are indicated. We did not observe drastically changed levels of 5S rRNA in our RNA gels even though in all constructions the gene is located distal to the 4.5S RNA gene. This is in agreement with previous findings that 5S RNA not incorporated into ribosomes is unstable (35). Major 4.5S RNA precursors are marked by dashes on the right side of the gel. The relative amounts of 4.5S RNA are summarized in Fig. 5.

lifetime of the hybrid mRNA or on its translatability. The effect of different flanking sequences on the lifetime of hybrid RNAs can also be observed in the 4.5S RNA system: with the exception of the P_1 precursor (Fig. 4, P_1P_2 lane) all other precursors are seen in constructions that include the

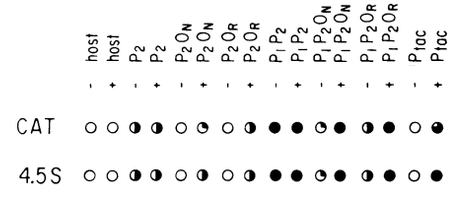

FIG. 5. Relative amounts of chloramphenicol acetyltransferase (CAT) and 4.5S RNA expression with different promoters. Several protein and RNA gels were scanned. The bands for chloramphenicol acetyltransferase and 4.5S RNA were brought in relationship to constant protein or RNA bands to correct for variations in cell density, gel backgrounds, pipetting, etc., as well as amounts of unprocessed transcripts in the various constructions. In each case, the value for the P_1P_2 construction (pAH1-1 and pKK290-2) was taken as 100%. Values obtained in the presence of IPTG (induced) are indicated (+). Full circles indicate values similar to the highest levels (P_1P_2). Open circles represent values similar to background levels (expression from the chromosomal 4.5S RNA gene). Half circles represent values corresponding to P_2 levels (45%–70% of P_1P_2 levels). One-quarter and three-quarter filled circles represent the respective intermediate levels.

lac operator in the natural orientation (P_2O_N, $P_1P_2O_N$, P_{tac}). These ladders of precursors (which are most likely multiple conformers of one RNA because only one band is seen on denaturing gels; not shown) are only found in traces in the corresponding constructions containing the operator in the reverse orientation (P_2O_R, $P_1P_2O_R$). The fact that the estimated sizes of the P_1 (≈270 nucleotides), P_2O_N (≈170 nucleotides), and P_{tac} (≈180 nucleotides) precursors correspond well with the numbers of nucleotides based on the known sequences calculated from the presumed initiation points of the various promoters up to the 3′ end of the 4.5S RNA gene (267, 169, and 179 nucleotides, respectively) indicates that these precursors most likely contain additional sequences 5′ to the 4.5S RNA gene. Since there is no correlation between the transcriptional activity and precursor levels (small amounts of precursor can be seen even in the uninduced P_{tac} construction; Fig. 4), these effects cannot be due to a saturation of host RNase P, but, rather are due to structural differences influencing the rate of 4.5S RNA maturation by RNase P, the processing enzyme (38).

Our results show that neighboring sequences can affect RNA maturation. Therefore, direct measurement of RNA production by summing the immature and the 4.5S RNA levels will be reliable, whereas protein determination could be misestimated by effects on initiation of translation.

The single promoter P_2 is well controlled by the operator in either orientation where we observe a 20- to 50-fold control. The experiment with the combined promoters shows that P_2 has about one-half of the combined activity. The repressed basal level would be accounted for as a readthrough, past the repressor, from P_1, insensitive to I^q or I^{sq}. These data confirm earlier studies (30), which showed that the *lac* repressor is only partially effective at blocking readthrough transcription.

The P_2 constructions we describe in this paper can be turned off in I^q strains with similar efficiency as the *tac* promoter but they exert about one-half of its activity. $P_1P_2O_N$ or $P_1P_2O_R$, on the other hand, are at least as active but cannot be regulated completely. Therefore, the synthetic operator will also be placed near P_1. This should enable us to repress the tandem *rrnB* promoters completely. Furthermore, we have experimental evidence that *rrnB* promoter fragments containing larger flanking regions than the basic tandem promoter unit used in this study exert a significantly increased strength (unpublished observations). We are currently adding these regions back to the operator-containing promoter units.

Because of their strength, their insensitivity toward premature termination, and their ability to be controlled via the *lac* operator, the rRNA promoters may be a useful system for the overproduction of foreign genes. The approach exploiting a synthetic *lac* operator for the regulation of promoters is particularly attractive because of its potential use in other less characterized expression hosts, where no strong inducible promoters are available. rRNA promoters, which we have shown to be very efficient even in the expression of non-rRNA genes can easily be detected and isolated, the *lac* operator can be introduced near the transcription start site(s), and regulation occurs with the *E. coli lacI* gene under a weaker host promoter (provided on the same plasmid or a helper vector). The only prerequisite is that the inducer is taken up by the host cell. In fact, while this manuscript was in preparation, Yansura and Henner (39) have shown that expression of genes in *Bacillus* can be regulated with a plasmid-encoded *E. coli lacI* gene and an *E. coli lac* operator placed near a *Bacillus* promoter.

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