Expression of \( lacZ \) from the Promoter of the \( Escherichia coli \) spc Operon Cloned into Vectors Carrying the W205 \( trp-lac \) Fusion

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The expression of \( lacZ \) has been analyzed and compared in a series of promoter cloning vectors by measuring the amount of \( lacZ \) mRNA by hybridization and the amount of \( \beta \)-galactosidase by standard enzymatic assay. Expression was driven by the promoter, \( P_{spec} \), of the \( spc \) ribosomal protein operon. The vectors contained either the standard W205 \( trp-lac \) fusion with the \( trp \) operon transcription terminator, \( trpt \), located in the \( lacZ \) leader sequence, or a deletion derivative that functionally inactivates \( trpt \). In the presence of \( trpt \), \( lacZ \) expression was temperature dependent so that increasing the growth temperature reduced the accumulation of \( lacZ \) mRNA and \( \beta \)-galactosidase activity. The frequency of transcript termination at \( trpt \) was estimated to be near zero at 20°C and at about 45% at 37°C. The amount of \( P_{spec} \)-derived \( lacZ \) mRNA and the amount of \( \beta \)-galactosidase produced per \( lacZ \) mRNA varied, depending on the mRNA 5' leader sequence between \( P_{spec} \) and \( lacZ \). These results demonstrate that the quantitative assessment of promoter activities with promoter cloning vectors requires careful analysis and interpretation. One particular construct without \( trpt \) did not seem to contain fortuitous transcription or translation signals generated at the fusion junction. In this strain, \( lacZ \) expression from \( P_{spec} \) was compared at the enzyme activity and mRNA levels with a previously constructed strain in which \( lacZ \) was located 250 bp downstream of \( trpt \). At any given growth rate, the different activities of \( \beta \)-galactosidase in these two strains were found to reflect the same differences in their amounts of \( lacZ \) mRNA. Assuming that the promoter-\( lacZ \) fusions in these strains reflect the properties of the promoters in their normal chromosomal setting, it was possible to estimate the absolute transcription activity of \( P_{spec} \) and the relative translation efficiency of \( P_{spec}-lacZ \) mRNA at different growth rates. Transcription from the \( spc \) promoter was found to increase from about 10 transcripts per min at a growth rate of 1.0 doublings/h to a maximum plateau of about 23 transcripts per min at growth rates above 1.5 doublings/h. The translation frequency of \( lacZ \) mRNA expressed from \( P_{spec} \) was unaffected by growth rates.

To study gene regulation in bacteria and to define the basic properties of a promoter isolated from its normal control sites, the promoter of interest is often linked on a plasmid or phage vector to a reporter gene such as \( lacZ \). The promoter activity in \( lacZ \)-based vectors is then assessed from measurements of \( \beta \)-galactosidase, an enzyme that is very stable and easy to assay. The amount of enzyme produced depends in part on the mRNA, and in part on other factors, including the termination or antitermination properties of the transcribing RNA polymerase, the stability of \( lacZ \) mRNA, and the ability of the \( lacZ \) mRNA to compete with bulk mRNA for the initiation of translation. The latter factors may be affected by particular, unnatural sequence combinations at the junction of the operon fusion. In the work described below, we have attempted to identify and quantify some of these effects by measuring with various \( lacZ \) vectors the amounts of \( lacZ \) mRNA and of \( \beta \)-galactosidase produced from the promoter of the \( spc \) ribosomal protein operon.

Two widely used \( lac \)-based promoter cloning vectors, the phage ARS205 (3) and the plasmid pRS415 (33), were derived from the \( trp-lac \) fusion W205, isolated by Mitchell et al. (25). In these vectors, the W205 fusion is located downstream of the promoter cloning site and contains the end of \( trpA \), followed by one of the two \( trp \) operon transcription terminators, \( trpt \) (29, 35, 36). Termination at \( trpt \) is independent of rho and occurs with an efficiency of about 37% in vivo (27) and of about 25% in vitro (36). The \( trpt \) element also serves as a pause or stop signal for 3'-to-5' exonucleolytic degradation of mRNA (27). The second, rho-dependent transcription terminator, \( trpt' \), is located 250 bp downstream of \( trpt \) in the normal \( trp \) operon (27). The \( trpt' \) element is not present in the W205 fusion.

Expression from plasmid-cloned genes is difficult to quantify because of plasmid copy number effects; therefore, expression of the \( lacZ \) reporter gene is often studied after integration of the promoter-\( lac \) fusion into the chromosome, e.g., by using the \( ARS205 \) system. Lysogens constructed with these phage vectors carry a temperature-sensitive repressor to aid in preparing phage lysates for moving the operon fusion from one strain to another. Because of lysis induction at higher temperature, such strains are grown and analyzed at 30°C. In our laboratory, an alternative system to study gene expression was developed in which the plasmid-borne promoter-\( lac \) fusion is flanked by sequences of the \( Escherichia coli \) maltose (\( mal \)) genes. This allows for insertion of the plasmid-constructed promoter-\( lac \) fusions into the \( mal \) locus of the chromosome by a double recombination event (16, 38). These strains can be grown at any temperature.

We have observed that promoter-\( lac \) operon fusions derived from the original W205 \( trp-lac \) fusion exhibit temperature-sensitive expression of \( \beta \)-galactosidase activity. The temperature sensitivity in expression was seen with a number of promoters, including the \( P_1 \) or \( P_2 \) promoters from \( rmb \), the replication primer promoter of plasmid pBR322, and the pro-

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moter of the \( spc \) ribosomal protein operon. We show here that the temperature sensitivity in \( lacZ \) expression is caused by the presence of the \( trpt \) transcription terminator within the W205 \( trp-lac \) fusion. In addition, we show that new sequence combinations generated at the fusion junction can have other dramatic effects on \( lacZ \) expression and therefore may adversely affect the quantitation of promoter activity. The analysis allowed us to identify and characterize a particular \( P_{spc-lacZ} \) construct in which these anomalous effects were minimal and absent and in which the expression of \( lacZ \) appears to reflect the properties of \( P_{spc} \) in its natural chromosomal setting.

**MATERIALS AND METHODS**

**Plasmids and strains.** The plasmids and bacterial strains used and details of their construction are presented in Tables 1 and 2. To delete \( trpt \) from plasmid pXZ09-B, a 2.3-kb fragment from this plasmid, spanning the region from the center of the \( trpt \) palindrome to a site beyond a unique \( SacI \) site within \( lacZ \), was amplified by PCR. One of the primers added a BamHI site at the end bordering the \( trpt \) terminator. After cleavage of the PCR product with BamHI and SacI, the resulting fragment was inserted between the BamHI and SacI sites of plasmid pXZ09-B, thereby deleting 50 bp of the \( trpt \) region, including the end of \( trpt \) and half of the \( trpt \) terminator repeat (Fig. 1). The resulting new plasmid without a functional \( trpt \) transcription terminator is pSL03. The correct deletion was verified by DNA sequencing.

A 110-bp \( spc \) promoter fragment (from \( -51 \) to \( +59 \) relative to the transcription start; Fig. 1) and a 504-bp fragment, carrying the \( spc \) promoter and the coding sequence of r-protein L14 (\( ppN \); from \( -51 \) to \( +453 \)), were obtained by PCR with appropriate primers by using \( xgal-E1 \) DNA (12) as a template and adding EcoRI and BamHI restriction sites at the ends. After cleavage with EcoRI and BamHI, these fragments were inserted between the EcoRI and BamHI sites of plasmid pXZ09-B and pSL03 to yield pSL02, pSL04, and pSL06 (Table 1).

All plasmids were constructed in duplicate with independently synthesized PCR products and checked for correct length of restriction fragments and enzyme activity. The promoter-\( lacZ \) fusions from these duplicate plasmids were first recombined into the \( mal \) locus of the \( recBC \) strain JC9387 (Table 2) as described previously (16). The \( P_{spc-lacZ} \) fusions were then transduced with phase P1 into the \( lac \) deletion strain HB181, selecting for kanamycin resistance (\( Km^\prime \)). The correct insertion was verified by streaking onto MacConkey maltose and MacConkey lactose plates (24). Before transduction, the HB181 recipient was positive for \( mal \) but lacked \( lac \); after transduction the strains lacked \( mal \) but were positive for \( lac \).

The absolute enzyme activities in bacteria transformed with plasmids pSL02 and pSL04 varied considerably from transformant to transformant, which probably resulted from variations in plasmid copy number. When the \( P_{spc-lacZ} \) fusions from different transformants (including plasmids with independently prepared PCR products) were recombined into the \( mal \) locus of the chromosome so that they were present as a single copy, there was no significant variation (less than 10%) in the \( \beta \)-galactosidase activity expressed from \( P_{spc} \) in replicate cultures and strains (Table 3). As a background control, the host strain was separately transformed with vector plasmids without promoter inserts. In pXZ09-B and pSL03 transformants, a low level of \( \beta \)-galactosidase activity is expressed from an upstream \( trp \) promoter. After recombination of \( lacZ \) from these plasmids into the chromosomal \( mal \) locus, the \( trp \) promoter is excluded and the resulting strains showed a \( lacZ \) activity near zero.

**Growth conditions.** Cultures were grown in Medium C (15) supplemented with either 0.2% (vol/vol) glycerol or 0.2% (wt/vol) glucose (with or without 0.8% Difco Casamino Acids plus 50 \( \mu \)g of tryptophan per ml), or they were grown in LB medium (24) with 0.2% glucose. Minimal media were supplemented with phenylalanine and threonine at 50 \( \mu \)g/ml. Experimental cultures were inoculated from overnight cultures in glycerol minimal medium by diluting at least 250-fold into minimal medium or 2,000-fold into amino acid-supplemented medium.

Growth was followed as the increase in turbidity at 600 nm with a 1-cm light path (i.e., the optical density at 600 nm \( [\text{OD}_{600}] \)). Since the turbidity is not exactly proportional to the culture density, the observed values, after subtraction of the medium blank, were corrected for nonlinearity (4). The corrected OD values deviated by less than 1% from the average exponential curve, so that the accuracy of the average OD used for determination of the specific enzyme activity was about 1%. For measurements of mRNA decay (see Fig. 5), rifampin was used at a concentration of 300 \( \mu \)g/ml and was added when the culture had reached an \( \text{OD}_{600} \) of about 0.32.

**Determination of \( \beta \)-galactosidase specific activities.** For determination of \( \beta \)-galactosidase content, several 10- or 20-ml samples were taken from an exponential culture over a period of two or three generations, and \( \beta \)-galactosidase was assayed as described earlier (38). The volume of the final reaction mixture before the addition of an equal volume of stopping solution was 1.0 ml. The assays were incubated at 37°C, generally for 50 to 90 min. Similar assays were performed with media blanks. The \( \beta \)-galactosidase activity was determined as the increase in \( A_{420} \).
of 0.1 mM EDTA in diethyl pyrocarbonate (DEPC)-treated water. After the UV
washed with high-salt-concentration and ethanol-containing buffers provided
ethanol, the lysate was filtered through the RNAqueous column by centrifuga-
resuspended in 0.3 ml of guanidinium thiocyanate lysis medium provided with
pelleted by low-speed centrifugation (5 min at 5,000 rpm) and homogeneously
phenol stopping solution (5% phenol in ethanol) at 22°C. The bacteria were
than 1.5:1 suggested minimal RNA degradation.
beginning of the
mentary to the 5
contained 25 ng of total RNA.

RESULTS
Features of promoter cloning vectors pRS415, pXZ09, pXZ09-B, and pSL03. Plasmids pRS415, pXZ09, pXZ09-B, and pSL03 (Table 1 and Fig. 1) are promoter cloning vectors, designed specifically to detect and measure promoter activities. The plasmid pRS415 (33) was derived from plasmid pBR322 and carries the W205 trp-lac fusion (25); its cloning site is located between four upstream rmb transcription terminators and a downstream promoterless lacZ gene. The DNA section between the cloning site and the start codon of lacZ consists of 75 bp of trp sequence (including the distal 28 bp of trpA and the 47-bp trailer sequence containing the trp operon transcription terminator, trpt; refs. 28, 37) and the 17-bp lacZ leader sequence (including the wild-type ribosome binding site, but not the promoter and operator sequences). Without an insert, this plasmid expresses very low background β-galactosidase activity; with an insert containing a promoter, β-galactosidase activity is elevated, but the extent of elevation is temperature sensitive. That is, β-galactosidase activity is substantially re-
duced at higher temperatures; this effect has been observed with several different promoters and has been attributed to the presence of trp in the cloning vector (see below).

Both pXZ09 and pXZ09-B carry the cloning site and the W205 trp-lacZ fusion of pRS415. In addition, these plasmids carry a lac gene and segments of the E. coli mal genes, which permit the recombinatorial insertional operon fusions con-
structed on the plasmids into the mal locus of the chromosome (16). Plasmid pXZ09-B was obtained from pXZ09 (38) by removing two BamHI sites flanking the kan gene; the remaining
BamHI site on pXZ09-B forms part of the multiple cloning site. The plasmid pSL03 (Fig. 1) was obtained from pXZ09-B by deleting 50 bp of trp sequences, beginning at BamHI of the cloning site and ending between the palindromic sequences that form the trp operon transcription terminator, trpt (37). By numerous criteria, this deletion appears to inactivate trpt and results in elevated expression of the downstream lacZ gene. Moreover, the temperature sensitivity of β-galactosidase expression was greatly reduced or eliminated by the deletional inactivation of trpt (see below).

Promoter constructs with and without trp and without rplN. To illustrate the effects of trpt and other features of operon fusions that might complicate the quantitation of promoter activity, two sets of promoter fusions were con-
structed by inserting DNA fragments containing the promoter for the spo ribosomal protein operon into pXZ09-B (with trpt) and into pSL03 (without trpt). In the first set, pSL02 and pSL04, the insert contained only the spo promoter (from nucleotides [nt] −51 to +59 relative to the transcription start site), whereas in the second set, pSL05 and pSL06, the insert contained the spo promoter and the first gene (rplN) of the spo operon (from nt −51 to +453; see Fig. 1). To eliminate plasmid copy number effects, the Pspo-lacZ fusions in pSL02 and pSL04 and the PSpo-rplN-lacZ fusions in pSL05 and pSL06 were recombined into the mal locus of the chromosome of the lacZ deletion strain HB181. With these chromosomal constructs (strains SL102, SL104, SL105, and SL106, respectively; Table 2), there was little variation in the amount of β-galactosidase specific activity in replicate constructs or replicate cultures (see standard deviations of enzyme activity in Table 3).

lacZ expression from Pspo at different temperatures. Cul-
tures of the four different strains, SL102 (Pspo-lacZ with trpt),
SL104 (P_{spc}-lacZ without trpt), SL105 (P_{spc}-rplN-lacZ with trpt), and SL106 (P_{spc}-rplN-lacZ without trpt) were grown at four different temperatures between 20 and 42°C. The β-galactosidase specific activities and the relative amounts of lacZ mRNA for the cultures were determined. In the two strains carrying the trpt element upstream of lacZ, expression of both lacZ mRNA and β-galactosidase enzyme was temperature sensitive (Fig. 2a through d, circles). However, enzyme activity and mRNA were not strictly proportional, as seen from the non-parallel curves in the semilog plots used. This suggests that the amount of β-galactosidase produced per amount of lacZ mRNA varies with the temperature. This is best illustrated by visualizing the ratio of β-galactosidase specific activity to the amount of lacZ mRNA as a function of temperature (Fig. 2e and f, circles). The values in these curves decrease with increasing temperature. This suggests that, in the presence of sequences associated with trpt, lacZ mRNA translation is severely more efficient at 20°C than at 42°C.

Removal of the transcription terminator trpt from the respective promoter lacZ fusions altered both the β-galactosidase specific activity and the amount of lacZ mRNA produced (Fig. 2a through d, triangles). In this case, the response of the enzyme activity was nearly proportional to that of the mRNA, so that the ratio curves (Fig. 2e and f, triangles) are essentially flat. These results indicate that the presence of trpt at the spc-lac junction directly or indirectly influences translation initiation at the downstream lacZ ribosome binding site in a temperature-dependent manner. Removal of trpt abrogates this effect. It is also apparent that the efficiency of translation of lacZ is influenced by 5' leader sequences in a temperature-independent manner. For example, the presence of the rplN sequence in the leader resulted in a twofold reduction in lacZ translation when the two fusions lacking a functional trpt are compared (Fig. 2e and f, triangles).

**Effect of different temperatures on transcript termination at trpt.** To visualize the effect of trpt on transcript termination at different temperatures, the ratio of observed transcripts +trpt/−trpt has been plotted as a function of growth temperature (Fig. 2g and h; obtained from the data in Fig. 2c and d). With or without rplN sequences, this ratio decreased with increasing temperature. This suggests that the efficiency of termination at trpt increases with increasing temperature. The value of this ratio was expected to be maximal 1.0 when trpt is totally inactive, and this ratio should be less than unity when any portion of the transcripts terminate at trpt. When rplN was present upstream of lacZ, the results were consistent with this expectation (Fig. 2h): termination at trpt was about 10% efficient at 20°C but more than 60% efficient at 42°C. Surprisingly, however, when rplN was absent, the ratio was greater than unity at low temperatures (2.2 during growth at 20°C; Fig. 2g). In this case the amount of lacZ transcript was increased, rather than decreased, by the presence of trpt. This suggests that the particular spc-trp-lac fusion without both trpt and rplN generates a fortuitous signal at the fusion junction that reduces the accu-

**TABLE 3. β-galactosidase specific activities expressed from the spc promoter**

<table>
<thead>
<tr>
<th>Strain</th>
<th>trpt</th>
<th>Promoter</th>
<th>rplN</th>
<th>Sp act</th>
<th>No. of cultures tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>SL103</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>0.38</td>
<td>2</td>
</tr>
<tr>
<td>SL102</td>
<td>+</td>
<td>P_{spc}</td>
<td>−</td>
<td>26 ± 2</td>
<td>14</td>
</tr>
<tr>
<td>SL104</td>
<td>−</td>
<td>P_{spc}</td>
<td>−</td>
<td>83 ± 8</td>
<td>11</td>
</tr>
<tr>
<td>SL105</td>
<td>+</td>
<td>P_{spc}</td>
<td>−</td>
<td>41 ± 3</td>
<td>2</td>
</tr>
<tr>
<td>SL106</td>
<td>−</td>
<td>P_{spc}</td>
<td>−</td>
<td>82 ± 8</td>
<td>3</td>
</tr>
</tbody>
</table>

a LB glucose medium at 37°C was used for these experiments. The presence (+) or absence (−) of features are indicated.

b The units for specific activity are A_{540} 420 per hour per OD_{600} unit (see Materials and Methods). Values given are averages and standard deviation.
ulation of transcripts, particularly at lower temperatures (Fig. 2e). This signal, although undefined, could either cause transcript termination or reduce transcript stability. When rplN sequences are present between P\textsubscript{psc} and the trp-lacZ fusion, indications of this additional signal were not present (Fig. 2h).

Effect of different growth rates on lacZ expression and termination at trpt. Using the same four strains, the effects of both (i) the presence or absence of trpt and (ii) the presence or absence of rplN on lacZ expression (enzyme and mRNA) were determined during growth at 37°C in different nutritional media (Table 4; Fig. 3). At all growth rates of between 1.0 and 3.0 doublings/h, the deletion of trpt resulted in the expected increase in lacZ mRNA total RNA and in β-galactosidase specific activity (e.g., compare strains SL104 and SL102 or strains SL106 and SL105 in Table 4 or Fig. 3). The simplest interpretation of this observation is that at all growth rates a fraction of transcripts initiated at P\textsubscript{psc} is terminated at trpt when it is present. Assuming that only the transcript termination function of trpt is responsible for the reduction in the amount of lacZ mRNA, the fraction of transcripts terminated was estimated by comparing the amounts of lacZ mRNA (per total RNA) in the two isogenic (+trpt/−trpt) strain pairs (i.e., strains SL102 and SL104 and strains SL105 and SL106). With one exception, the efficiency of transcript termination at trpt was estimated to be between 43 and 46% in both fast- and slow-growing cultures (Table 4). The exception occurred in the SL102-SL104 pair in glycerol medium, where a higher value of 74% was found. This higher value apparently results from an exceptionally high accumulation of lacZ mRNA in the reference strain SL104 without trpt (compare hybridization data in Fig. 3c and d), which may be related to the abnormality described above for this strain and illustrated in Fig. 2g. Therefore, in the absence of other complicating factors, the transcript termination function of trpt is probably not growth rate dependent.

With increasing growth rate, the β-galactosidase specific activity was not strictly proportional to the amount of lacZ mRNA (Fig. 3). The main reason for this is that the specific activity represents enzyme activity per culture mass, whereas the hybridization data represent transcripts per amount of total RNA. The two reference units, culture mass and total RNA, change differently with respect to the exponential growth rate (see below).

Comparison of lacZ expression from different promoters. Because of different sequences at the junction of the spc-trp-lac operon fusion, the β-galactosidase activities per amount of lacZ mRNA expressed from P\textsubscript{psc} were different in the four strains examined (Table 4). The results in Fig. 2g and h suggest that lacZ expression in strain SL106 (deletion of trpt; inclusion of rplN upstream of lacZ) was less influenced by artificial or fortuitous transcription and translation signals at or near the fusion junction than was expression in the other three strains. To test this supposition, lacZ expression from P\textsubscript{psc} in strain SL106 (P\textsubscript{psc}-rplN-lacZ) was compared with lacZ expression from the P1-P2 tandem promoters of the rmB operon by using strain NZ231 (rmB P1-P2-lacZ [38]). The rRNA promoters were chosen because their absolute activity is known and their importance for the control of ribosome synthesis has been established.

Expression from the rRNA promoters increased with increasing growth rate at both enzyme and mRNA levels (Fig. 4a and b, circles), whereas expression from P\textsubscript{psc} decreased at the enzyme level and was nearly constant at the mRNA level (Fig. 4a and b, triangles). Despite these differences, the amounts of β-galactosidase made per lacZ mRNA were essentially the same for both promoters (Fig. 4c, filled circles and triangles; the data are normalized for the different reference units as indicated in the figure legend). Thus, in these two strains, differences in β-galactosidase activity at a given growth rate were correctly reflected by differences in lacZ mRNA accumulation.

These values reflect the amount of β-galactosidase per lacZ mRNA; they may be multiplied with the rate of culture growth (ln2/τ, where τ is the culture doubling time) to obtain the relative rate of β-galactosidase accumulation per lacZ mRNA. This rate was approximately constant (Fig. 4c, open symbols). As anticipated, the translation per lacZ mRNA was independent of the promoter from which lacZ was expressed, i.e., rmB P1-P2 or P\textsubscript{psc} (Fig. 4c, circles and triangles, respectively).

Lifetime of lacZ mRNA. To find the relative rate of lacZ mRNA synthesis expressed from P\textsubscript{psc} and rmB P1-P2, the decay of lacZ mRNA was determined by following the disappearance of the lacZ hybridization signal during growth in the presence of the antibiotic rifampin (Fig. 5a and b, circles and triangles, respectively). The hybridization probe, a 308-bp section that includes the 5’ end of the lacZ coding region, was the same as in the preceding experiments. For comparison, the decay of lacZ mRNA expressed from its natural promoter, P\textsubscript{lac}, was observed with the isogenic strain HB123 (Table 2) carrying a wild-type lac operon (Fig. 5, diamonds). To obtain information about the functional life of lacZ mRNA under these conditions, the residual accumulation of β-galactosidase during rifampin treatment was also measured (Fig. 5c and d). The cultures were grown in either glycerol minimal or LB medium (Fig. 5, left and right panels). In all three strains (i.e., SL106, NZ231, and HB123) the accumulation of total RNA (mainly rRNA) stopped immediately after the addition of rifampin (Fig. 5d, open symbols), indicating that the strains used were rifampin sensitive.

After the addition of rifampin to cultures grown in glycerol minimal medium, lacZ mRNA expressed from P\textsubscript{psc} and rmB P1-P2 decayed initially at about the same rate, corresponding to an average life of 1.8 min (Fig. 5a). However, for P\textsubscript{psc}-derived mRNA, the decay slowed down after 1 min, leading to a plateau of apparently stable mRNA (i.e., of the 5’-terminal region of lacZ mRNA) at about 40% of the zero time (exponential growth) level (Fig. 5a). In LB medium, the initial decay rates appear to be slightly lower than in the minimal medium (about a 2.4-min average life), and the plateau of P\textsubscript{psc}-derived mRNA was at about 64% of the zero time level (Fig. 5b). The decay of rmB P1-P2-derived mRNA also slowed. In this rich medium, the stability of P\textsubscript{psc}- and rmB P1-P2-derived lacZ mRNA was reflected in a continuing synthesis of β-galactosidase in the presence of rifampin at a rate corresponding to 15% of the enzyme synthesis rate observed immediately before the addition of rifampin (Fig. 5d). We cannot explain why lacZ mRNA from heterologous promoter constructs fails to decay completely in the presence of rifampin (see Discussion).

In control experiments, mRNA expressed from the lac operon promoter, P\textsubscript{lac}, decayed exponentially and apparently completely (Fig. 5a and b, diamonds). The decay rates for P\textsubscript{psc}-derived mRNA were identical to the initial decay rates observed for P\textsubscript{psc} and rmB P1-P2-derived mRNAs in the two media. We assume that these rates (ca. 1.8 min in glycerol medium and 2.4 min in LB medium) reflect the decay rate of lac mRNA during exponential growth.

In further control experiments (unpublished data), the decay of rplN and rplX mRNA (first and second genes in the spc operon) was measured in the strains HB123 and SL106 with appropriate probes. These mRNAs, when derived from the spc operon, decayed exponentially and identically in the two strains used (the same RNA preparations were used as for Fig. 4c).
specific activities (ratio of the two curves in Fig. 4a). Either absence of trpt recombined into the mal locus were used: SL102 (P<sup>spc</sup>-trpt-lacZ, left panels, @); SL104 (P<sup>spc</sup>-lacZ, left panels, □); SL105 (P<sup>spc</sup>-rplN-trpt-lacZ, right panels, △); and SL106 (P<sup>spc</sup>-rplN-lacZ, right panels, ●). Cultures were grown at different temperatures in LB medium supplemented with glucose, and β-galactosidase specific activities (panels a and b) and lacZ mRNA per total RNA (panels c and d) were measured. The ratio of β-galactosidase specific activity and lacZ mRNA per total RNA (panels e and f) is a measure for the translation efficiency of lacZ mRNA (from the data in panels a and c or in panels b and d, respectively). The ratios of the amounts of lacZ mRNA observed in the presence or absence of a functional trpt (panels g and h, ●) at temperatures between 20 and 42°C are illustrated.

5). This indicates that the spc promoter is not resistant to rifampin inhibition and that the construction of SL106 did not cause a special mutation that affects mRNA decay. Therefore, the stabilization of mRNA from the spc-lac fusion appears to be specific for the fusion mRNA. It might reflect some special properties of r-protein mRNAs with respect to the control of their decay rates and the absence of those control sites in the fusion mRNA (see Discussion).

Absolute activity of the spc promoter. The activity of P<sub>spc</sub> relative to the combined activity of the two rRNA promoters rrnB P1-P2 was obtained as the ratio of either the amounts of lacZ mRNA expressed from P<sub>spc</sub> and rrnB P1-P2 (ratio of the two curves in Fig. 4b) or the corresponding β-galactosidase specific activities (ratio of the two curves in Fig. 4a). Either ratio decreased with increasing growth rate from about 1.5 at a growth rate of 1.0 doubling/h to about 0.3 at a growth rate of 3.0 doublings/h (Fig. 6a, circles and triangles, respectively). The results shown in Fig. 5 suggest that P<sub>spc</sub> - and rrnP1-P2-derived lac mRNA decayed at essentially equal rates during exponential growth so that, for a given medium, the different amounts of P<sub>spc</sub>- and rrnB P1-P2-derived lacZ mRNAs reflect the differences in their relative synthesis rates. The absolute

![FIG. 3. Growth rate dependency of lacZ expression from P<sub>spc</sub>. Four strains containing P<sub>spc</sub> promoter fusions recombined into the mal locus were used: SL102 (P<sup>spc</sup>-trpt-lacZ, left panels, ●); SL104 (P<sup>spc</sup>-lacZ, left panels, □); SL105 (P<sup>spc</sup>-rplN-trpt-lacZ, right panels, △); and SL106 (P<sup>spc</sup>-rplN-lacZ, right panels, ●). The media used to give increasing growth rates were glycerol minimal, glucose minimal, glucose-amino acids, and LB medium supplemented with glucose. All cultures were grown at 37°C, and culture growth rates, β-galactosidase specific activities, and lacZ mRNA per total RNA were measured.

![TABLE 4. Expression of lacZ from the spc ribosomal protein promoter in different strains grown at 37°C in LB medium or glycerol minimal medium](image)

<table>
<thead>
<tr>
<th>Medium and strain insert</th>
<th>β-Gal sp act&lt;sup&gt;a&lt;/sup&gt;</th>
<th>lacZ&lt;sub&gt;b&lt;/sub&gt; mRNA</th>
<th>β-Gal sp act/&lt;br&gt;mRNA&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Termination&lt;sup&gt;c&lt;/sup&gt; (fraction)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LB medium</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>SL102 P&lt;sup&gt;spc&lt;/sup&gt;-trpt-lacZ</td>
<td>23 ± 1</td>
<td>29 ± 2</td>
<td>0.79</td>
<td>0.43</td>
</tr>
<tr>
<td>SL104 P&lt;sup&gt;spc&lt;/sup&gt;-lacZ</td>
<td>89 ± 2</td>
<td>52 ± 1</td>
<td>1.71</td>
<td></td>
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<tr>
<td>SL105 P&lt;sup&gt;spc&lt;/sup&gt;-rplN-trpt-lacZ</td>
<td>36 ± 2</td>
<td>57 ± 4</td>
<td>0.63</td>
<td>0.43</td>
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<tr>
<td>SL106 P&lt;sup&gt;spc&lt;/sup&gt;-rplN-lacZ</td>
<td>79 ± 1</td>
<td>100 ± 7</td>
<td>0.79</td>
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<tr>
<td><strong>Glycerol minimal medium</strong></td>
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<tr>
<td>SL102 P&lt;sup&gt;spc&lt;/sup&gt;-trpt-lacZ</td>
<td>25 ± 1</td>
<td>29 ± 2</td>
<td>0.86</td>
<td>0.74</td>
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<tr>
<td>SL104 P&lt;sup&gt;spc&lt;/sup&gt;-lacZ</td>
<td>298 ± 5</td>
<td>108 ± 10</td>
<td>2.75</td>
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<tr>
<td>SL105 P&lt;sup&gt;spc&lt;/sup&gt;-rplN-trpt-lacZ</td>
<td>59 ± 1</td>
<td>52 ± 9</td>
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<td>0.46</td>
</tr>
<tr>
<td>SL106 P&lt;sup&gt;spc&lt;/sup&gt;-rplN-lacZ</td>
<td>195 ± 2</td>
<td>97 ± 11</td>
<td>2.01</td>
<td></td>
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<sup>a</sup>Measured as the increase in A<sub>405</sub> per hour per OD<sub>600</sub> unit of culture mass present in the β-galactosidase (β-Gal) assay. For each of the eight cultures (four strains in two media each), two enzyme assays were performed about one generation time apart before samples for RNA preparation were taken.

<sup>b</sup>Amount of 5’-terminal lacZ mRNA in relative units per amount of total RNA. The standard deviation was obtained from four to five hybridization experiments (see Materials and Methods).

<sup>c</sup>Ratio of average β-galactosidase specific activity to the average relative amount of lacZ mRNA per total RNA.

<sup>d</sup>One minus the ratio of lacZ mRNA observed with trpt present to that observed without trpt.

<sup>e</sup>The average hybridization values for strain SL106 grown in LB medium were set at 100 for normalization of all other hybridization values in this table.
activity of the rRNA promoters in rRNA transcripts per minute was determined previously (4, 6) and is illustrated in Fig. 6b (open symbols). The rRNA gene activity increases from about 3 to over 60 initiations/min in the range between 0.6 and 3.0 doublings/h. By multiplying the rRNA gene activity with the relative P

lacZ

activity (relative to the rRNA P1-P2 activity), absolute P

lacZ

activities were obtained (Fig. 6b, circles).

FIG. 4. Comparison of lacZ expression from P

lacZ

and from the P1 and P2 promoters of rmb. Two strains, SL106 (P

spc-rplN-lacZ, ▲) and XZ231 (rmb P1-P2-lacZ, ●), were grown in four different media supporting growth rates of between 1.0 and 3.0 doublings/h (see legend to Fig. 3). Two samples were removed from each culture for measurement of β-galactosidase specific activity (panel a), and one sample was removed for preparation of total RNA. Each RNA preparation was used in two independent hybridization assays for determination of lacZ mRNA per total RNA (panel b). The β-galactosidase activity per mRNA (panel c, solid symbols) was obtained in relative units by first forming the ratio of the data in panels a and b and then dividing this quotient by the RNA/OD600 values were 5.8 x 10^13, 6.6 x 10^13, 9.4 x 10^13, and 10.7 x 10^13 RNA nucleotides per OD U, respectively (4, 6). The division by these values corrects for the different reference units used for enzyme specific activity (OD600) and hybridization (total RNA). The rate of translation per lacZ mRNA (panel c, open symbols) was obtained by multiplying the data represented by the solid symbols by the growth rate (ln2/τ).

DISCUSSION

Transcription termination and other effects of trp. The expression of β-galactosidase activity from promoter cloning vectors based on the classical W205 trp-lac fusion has been used in the past to study promoter activities under different physiological conditions. Generally, the promoter-lacZ fusions are integrated into the bacterial chromosome by using phage λ vectors (3), and the resulting lysogens are grown at 30°C because of the presence of a temperature-sensitive λ repressor. rmb promoters have been studied by using the mal chromosomal integration system (16) at 37°C (38, 39), which is the standard temperature for physiological experiments with E. coli. However, when we tried to use this system to study the pBR322 replication primer promoter, we noticed that the β-galactosidase activity expressed from the primer promoter was temperature sensitive (unpublished observations). Subsequently this temperature sensitivity was confirmed with other promoters, including the rmb P1 and P2 promoters and the ribosomal protein P

spc

promoter (unpublished observations). Here we have traced the temperature sensitivity to the presence of the rho-independent trp transcriptional terminator that is located immediately upstream of lacZ in the W205 trp-lac fusion (Fig. 1).

The frequency of transcription termination at trp was estimated by comparing the amount of lacZ mRNA present in isogenic P

spc-rplN-lacZ and SL105 (P

spc-rplN-trp-lacZ) to that obtained in the SL106 (P

spc-rplN-lacZ) strain (Fig. 4c) in which the P1-P2 tandem promoters of rmb were deleted in pSL03 (Fig. 1b) produces a temperature-dependent mRNA stability signal that causes the observed temperature effects.

A comparison of lacZ expression at the enzyme and mRNA levels in isogenic strain constructs with or without trp and with or without an rplN sequence in the lacZ leader revealed a number of anomalously low results that remain uncharacterized. We suggest that these anomalies are due to artificial sequence combinations at the spe-trp-lac fusion junction and that they influence features such as termination and antitermination properties of the lacZ gene. These indirect effects defeat the purpose of promoter-lacZ fusions, i.e., to obtain information about the promoter activity. We therefore focused our attention on the P

spc

fusion containing rplN and lacking trp. In this strain (SL106), such anomalies effects appeared to be minimal if not completely absent.

Expression of β-galactosidase from P

spc and P1-P2

trp. In the strain SL106 (P

spc-rplN-lacZ without trp), the β-galactosidase activities per amount of lacZ mRNA observed at different growth rates were the same as in a previously constructed strain XZ231 (Fig. 4c) in which the P1-P2 tandem promoters of the rmb operon were deleted and lacZ was linked to lacZ (38). The rmb P1-P2-lacZ fusion includes trp but, in addition, contains the antitermination elements of rRNA genes which are assumed to prevent or at least greatly reduce termination at trp: the read-
through at trp at 30°C has been reported to be fourfold increased, from 17 to 73%, by the presence of the rrnE antitermination sites (1). The rrnB P1-P2-lacZ fusion also includes 1,120 bp of phage λ DNA between the rrnB P1-P2 promoters and the trp-lacZ section. Insertion of this DNA “spacer” was necessary for the initial cloning of strong rRNA promoters on pBR322-derived plasmids (38). The λ DNA sequences inserted do not contain known promoters or ribosome binding sites. The observation that the differences in β-galactosidase synthesis in the strains SL106 and XZ231 correctly reflect the differences in the accumulation of 5\(^9\)-terminal lacZ mRNA (Fig. 4c) suggests that fortuitous translation signals at the trp-lac fusion junction are either the same or absent for these two constructs. The anti-transcription termination sites of the rRNA promoters apparently do not affect the expression of β-galactosidase activity by suppressing polarity within lacZ. Furthermore, the initial decay rates of lacZ mRNA after the addition of rifampin were about the same in SL106 and XZ231 (Fig. 5a and b), suggesting that the different lacZ leaders in these two strains do not differently affect lacZ mRNA decay during exponential growth; at later times after rifampin addition lacZ mRNA decay was clearly different. For these reasons, the use of the rm P1-P2-lacZ construct in XZ231 as a reference for comparison with the P\(_{spc}\)-rplN-lacZ fusion in SL106 appears to be justified, despite the differences in the leader regions.

The β-galactosidase specific activity expressed from rmB P1-P2 in strain XZ231 increased with increasing growth rate (Fig. 4a, circles), as was expected in view of the increased ribosome synthesis at high growth rates and in agreement with previous reports (38). On the other hand, the β-galactosidase specific activity expressed from P\(_{spc}\) in strain SL106 decreased with increasing growth rate (Fig. 3b and Fig. 4a, triangles). A decreasing β-galactosidase expression from r-protein promoters with increasing growth rate is in contrast to the increasing amounts of r protein made per total protein (\(\alpha\) [8, 10, 32]). A similar decrease β-galactosidase specific activity with increasing growth rate has been observed previously with the promoter of another major r-protein operon, S10, linked to lacZ (19). As had been suggested in that study, it is possible that this discrepancy results from the omission on the fusion constructs of certain control sites located distally in these operons. These sites are thought to regulate the decay of r-protein mRNA via translational repression, translational coupling, and endonucleolytic cleavage followed by \(3\)'-to-\(5\)' exonucleolytic mRNA degradation (“retroregulation”; for a review, see reference 18). In the spc operon, such sites have been located downstream of rplN (22) and are expected to stabilize the mRNA when the production of 16S rRNA exceeds or equals the production of the S8 regulatory protein. Based on the arguments above, we suggest that, in the absence of fortuitous transcription and trans-
The rate of spe mRNA synthesis relative to the rate of total mRNA synthesis ($r_{spe}/r_{mRNA}$) has been reported to increase with increasing growth rate similar to $\alpha$, (13, 21). This has suggested that r-protein synthesis is primarily regulated at the transcriptional level, so that the translational regulation only provides a “fine-tuning” to accurately adjust r-protein synthesis to rRNA synthesis (6, 13, 21). This interpretation was based on the plausible but unproven assumption that the rates of translation and degradation of bulk mRNA change with growth rate in a way similar to that of translation and degradation of spe mRNA. However, this assumption may not be warranted, especially since the rate of spe mRNA degradation appears to be subject to a special regulation dependent on the synthesis of rRNA (22).

The absolute activity of $P_{spe}$ was estimated above in transcripts initiated per minute per promoter by comparison with the known absolute activity of RNA promoters. At low growth rates, the $P_{spe}$ activity increased approximately in proportion to the rRNA promoter activity and then became constant above 1.5 doublings/h at about 23 transcripts/min (Fig. 6b). In view of the 1.1 kb of $\lambda$ DNA spacer between the promoters and lacZ in the operon fusion on strain XZ231, it seems possible that a fraction of the transcripts originating at $rrnB$ P1-P2 terminates before reaching lacZ. In that case transcription from $rrnB$ P1-P2 in strain XZ231 would be underestimated, so that the $P_{spe}$ activities in Fig. 6b would be overestimates. However, because of the transcription antitermination elements associated with the rRNA promoters, this may not be significant, so that the spe mRNA activities in Fig. 6b should be essentially correct.

Decay of lacZ mRNA in the presence of rifampin. An attempt was made to determine the average lifetime of $P_{spe}$- and $rrnB$ P1-P2-derived lacZ mRNA sequences by using rifampin to inhibit transcription initiation. Surprisingly, in the presence of rifampin lacZ mRNA expressed from $P_{spe}$ did not completely disappear (Fig. 5b), and in LB medium some residual $\beta$-galactosidase synthesis from $P_{spe}$ and $rrnB$ P1-P2 continued (Fig. 5d). A number of control experiments demonstrated that the rifampin used was fully active and that the bacterial strains were fully sensitive. First, the accumulation of stable RNA (rRNA and tRNA) ceased immediately after the addition of rifampin in all strains used (Fig. 5d). Second, lacZ mRNA derived from the lactose operon promoter in the isogenic strain HB123 decayed exponentially and completely in the presence of rifampin (Fig. 5a and b). Third, spe mRNA sequences derived from transcription of the spe operon in the spe-lac fusion strain decayed exponentially and completely in the presence of rifampin (data not shown). Finally, in the presence of rifampin, all bacterial cultures stopped growth immediately and non accumulated rifampin-resistant bacteria (data not shown). We therefore conclude that initiation of all RNA chains ceased in the presence of rifampin and that the incomplete or nonexponential decay of $P_{spe}$ and $rrnB$ P1-P2-derived lacZ mRNA observed at later times was due to mRNA stabilization. The mechanism responsible for this stabilization of fusion mRNA is not known. In part, it might be caused by a crowding of mRNA with ribosomes when bulk mRNA gradually vanishes during rifampin treatment. Similar decreased rates of mRNA decay as a result of ribosome crowding have been reported (23, 28). Conversely, when translation of the mRNA was reduced, the mRNA decay data of Fig. 5) and the different reference units were taken into account the decrease becomes somewhat greater than 20%. However, because of the differences in methods, growth media, and hybridization probes, these data sets are not strictly comparable and it is not clear whether the modest discrepancy between them is significant.
rate of lacZ mRNA decay has been found to increase (17). Again, this indicates that increased translation can result in a decreased rate of mRNA decay.

Based on the preceding arguments, we assume that the initial decay rates in the presence of rifampin reflect the decay rates during balanced exponential growth. The initial decay kinetics of lacZ mRNA derived from P_lac, P_{spc}, and rnb P1-P2 for a given medium were virtually identical. For glycerol medium, the initial rate corresponded to an average lifetime of about 1.8 min and for LB medium it was about 2.4 min. Since in a given medium the decay rates for P_{spc} and rnb P1-P2-derived mRNAs were the same, we were able to estimate the absolute activity of P_{spc} from the observed accumulations of lacZ mRNA (see above).

Features of new cloning vector pSL03. When a reporter system is used it does not seem prudent to include a transcription termination signal upstream of the reporter gene, particularly if the terminator activity is variable and affected by conditions such as temperature and growth media. For these reasons, we and other investigators (20) have removed trp from W205-derived vectors. The presence of trp in the W205 fusion might not have been apparent to all previous investigators; for example, when λRS205 was used as a cloning vector for P_sp by Miura et al. (26), they stated that the fusion W205 removes the transcription termination signal of the trp operon. Clearly, trp' was removed but trp was not.

In addition to the higher expression values due to the absence of the transcription terminator, pSL03 has several other desirable features. (i) In contrast to phage λ-based vectors with a temperature-sensitive repressor, the mal-inserted constructs can be grown at any temperature. Although λ-based vectors with a temperature-independent repressor are available, the presence of the prophage may not always be desirable. (ii) In the absence of a cloned promoter, there is very little background β-galactosidase activity when the construct is integrated into the chromosome. (iii) The location of mal close to oric on the E. coli chromosome produces a relatively constant gene dosage (11), in contrast to λatt near the middle of the E. coli replicon, which shows considerable changes in gene dosage at different growth rates (4). (iv) The orientation of the lacZ insertion into the chromosome at the mal locus is such that the direction of transcription and replication are aligned. This may be an advantage for active promoters since most operons with strong promoters are oriented in this manner.

A number of investigators (see, for example, reference 30) have also observed that in fusion constructs the translation of the reporter gene may be affected by fortuitous signals that arise at the junction of the fused operons. Linn and St. Pierre attempted to alleviate this problem by including in their vector an RNase III cleavage site upstream of lacZ so that all reporter gene mRNAs had the same 5' terminus (20). However, it is not certain even with their vector that RNase III cleavage and RNA polymerase transcription termination-antitermination properties are completely independent of growth conditions and sequences at the fusion junction. Therefore, for accurate quantitation a careful analysis of fusion gene expression is necessary with any vector.

ACKNOWLEDGMENTS
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REFERENCES
6. Bremer, H., and P. P. Dennis. 1996. Modulation of chemical composition and other parameters of the cell by growth rate, ppGpp is used it does not seem prudent to include a transcription