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Cloning and analysis of strong promoters is made possible by the downstream placement of a RNA termination signal

(promoter efficiency/RNA synthesis/RNA polymerase/gene expression)

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ABSTRACT **Downstream placement of a strong transcriptional termination signal has made possible the cloning of bacteriophage T5 promoters known to exhibit high signal strength. The cloning system constructed contains two easily assayable indicator functions whose expression is controlled by the integration of promoters and terminators, respectively. By assessing transcription within the indicator regions, the efficiency of promoters as well as termination signals can be determined *in vitro* and *in vivo*.**

The efficiency of interaction between *Escherichia coli* RNA polymerase and transcriptional promoters of *E. coli* varies within a wide range when measured *in vitro* (1). For unregulated promoters, the rate of complex formation *in vitro* reflects promoter strength *in vivo* (1, 2). However, despite the identification of more than 80 different promoter sequences and extensive study of promoter–RNA polymerase interactions (for survey, see refs. 3–5), the contribution of specific structural features to the functional activity of such sequences is not understood.

Promoters from various bacterial and viral sources have been cloned in *E. coli*, and their signal strength *in vivo* has been studied by using expression from distal promoterless sequences encoding β-galactosidase (β-Gal) or other proteins (6, 7) as an indicator of promoter activity. Attempts to clone small DNA fragments carrying the strong promoters of bacteriophage T5, which *in vitro* far exceed other promoters in the rate of complex formation with RNA polymerase and the rate of initiation of RNA synthesis (1, 2), have been unsuccessful; however, fragments of T5 DNA containing both a strong promoter and a strong termination signal have been cloned (8). Subsequently, electron microscope analysis has shown that transcriptional regions of several *E. coli* plasmids are organized in well-defined units where termination signals appear to balance transcription initiated at promoters of different strengths (9). Together, these findings suggested that the cloning of strong promoter signals from phage T5 or other sources might require the downstream placement of comparably strong termination signals.

We report here the construction and analysis of bacterial plasmid vectors that enable the cloning of promoters of high signal strength; such cloning is made possible by the positioning of a transcriptional termination signal downstream from the site of insertion of such promoters. The constructed plasmids, which allow estimation of the strength of promoter signals *in vitro* and *in vivo*, contain indicator genes in positions that also permit selection for termination signals. Using these vectors, we have isolated a library of T5 promoter sequences suitable for biochemical and physical investigations of promoter function and also potentially useful for achieving high-level transcription of heterologous genes introduced distal to the promoter signals.

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MATERIALS AND METHODS

Restriction endonucleases, *E. coli* DNA polymerase, and bacteriophage T4 DNA ligase were purchased from several commercial sources, and reactions were carried out as suggested by the supplier. *Eco*RI synthetic linker and adapter sequences were obtained from Collaborative Research (Waltham, MA). Phage *fd* DNA (replicative form) and plasmid pAD16/30 containing a 28-base-pair (bp) *Hin*dIII/*Bam*HI adapter sequence were gifts from H. Schaller. *lac* repressor was a gift from A. Riggs. Isolation of bacteriophage T5 DNA (2), plasmid DNA (10), *E. coli* RNA polymerase (2), and termination factor *rho* (11) have been described previously.

The binding of RNA polymerase to promoters and subsequent analysis of the complexes by nitrocellulose filter binding have been described (2). Identification and isolation of *lac* operator-containing DNA fragments by repressor binding utilized the procedure of Riggs *et al.* (12). The conversion of protruding 5′ single-stranded DNA extensions to blunt ends and DNA ligation reactions have been described (13). Synthetic linker and adapter sequences were present in 3- to 10-fold excess relative to the various DNA fragments. Transformation of *E. coli* strains $C600r^-m^+$ (our laboratory collection), the M15 deletion-mutant DZ 291 (obtained from A. V. Fowler), and BMH71-18, an M15 derivative carrying the $lacI^q$ mutation (obtained from B. Mueller-Hill), was carried out as described (14).

Selection of transformants involved plating on LB plates containing chloramphenicol (Cm, 20 μg/ml), ampicillin (Ap, 100 μg/ml), or varying amounts of tetracycline (Tc; 2–70 μg/ml). Selection for presence of the *lac* operator or production of the α fragment of β-Gal was carried out on plates containing the antibiotic plus 5-bromo-4-chloro-3-indolyl β-*O*-galactoside at 40 μg/ml (15). Induction of *lac* expression by isopropylthiogalactoside was as described (15). *In vitro* and *in vivo* RNA was prepared and analyzed as described previously (1) except that [α-^{32}P]UTP and [^{32}P]phosphate were used for labeling *in vitro* and *in vivo*, respectively. *In vivo* RNA was isolated from plasmid-containing C600 cells after a 10-min labeling period.

RESULTS

Experimental Strategy. We have constructed a family of plasmids (Fig. 1) that carry two DNA segments that can be brought under the control of a single promoter and are separated by an endonuclease cleavage site suitable for the cloning

Abbreviations: bp, base pair(s); Cm, chloramphenicol; Tc, tetracycline; Ap, ampicillin; β-Gal, β-galactosidase.

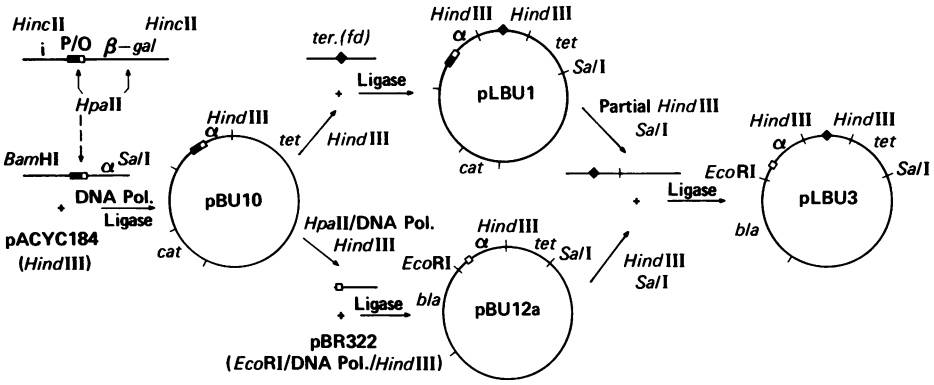

FIG. 1. Construction of plasmids for cloning terminators and promoters. A 780-bp DNA fragment carrying the *E. coli lac* regulatory region (promoter/operator: P/O), an NH_2-terminal portion of the β-Gal structural gene (*β-gal*) sufficient for intracistronic complementation of the M15 deletion, and a portion of the *lac*I-gene (I) was isolated from a *Hinc*II digest of pACYC214 plasmid (unpublished data) DNA by repressor binding and subsequent adsorption to nitrocellulose (16). By using the *Hpa* II cleavage site within *β-gal*, the fragment was reduced in size and provided with *Bam*HI and *Sal* I termini by various subclonings (pBU8, pBACH16). The resulting fragment (leftmost part of the figure) contains the intact control region of the *lac* operon and a portion of the *β-gal* structural gene encoding 66 NH_2-terminal amino acids (α). Introduction of this fragment, by blunt-end ligation, into the *Hin*dIII site of pACYC184 (17) yielded pBU10, a vector suitable for terminator cloning. The major terminator of the coliphage *fd* genome was isolated as a 338-bp *Sau*3A fragment (18), ligated to a *Bam*HI/*Hin*dIII adaptor sequence, and introduced into pBU10 to yield pLBU1. Cleavage of the *lac* sequence in pBU10 by *Hpa* II endonuclease destroyed the *lac* promoter and, upon cleavage with *Hin*dIII, liberated a fragment containing the *lac* operator and a region coding for functional α fragment. Integrating this DNA sequence into pBR322 (19) led to pBU12a. Finally, replacement of the *Hin*dIII/*Sal* I portion of pBU12a by the *Hin*dIII/*Sal* I fragment of pLBU1 containing the *fd* terminator resulted in pBLU3, a vector suitable for the cloning of efficient promoters at the *Eco*RI site. The regions encoding Cm, Ap, and Tc resistance are indicated as *cat*, *bla*, and *tet*, respectively.

of transcription termination signals. In the absence of an intervening terminator, the level of transcription of both segments is identical and reflects the efficiency of the upstream promoter; reduced transcription of the distal DNA segment upon insertion of a transcription terminator reflects termination efficiency.

To assay the level of transcription, we chose DNA segments that contain genes encoding β-galactosidase (*β-gal*) activity and Tc resistance. Because the NH_2-terminal polypeptide (α fragment) can restore β-Gal activity in *lac* M15 deletion mutants (20), we used just the portion of the *lac* operon that produces a functional α fragment.

By using efficient termination signals, we expected to obtain the stable integration of DNA fragments containing strong promoters; these could be identified by the relatively high level of Tc resistance that resulted from transcriptional read-through past the terminator into the distal *tet* region.

Construction of Plasmid Vectors. A vehicle for the selective insertion of transcription terminators between the two indicator genes was obtained by fusing a 560-bp fragment carrying the *lac* promoter-operator region and part of the *lac*Z structural gene to the *tet* region of pACYC184 (Fig. 1). The resulting plasmid, pBU10, has the following properties (Table 1; Fig. 2): (*i*) it complements the M15 deletion of the *lac* operon; (*ii*) the Tc resistance it specifies is under control of the *lac* promoter, as shown in M15*lacI*^{q} strains; (*iii*) the *Hin*dIII cleavage site between the *lac* gene fragment and the *tet* gene is restored; (*iv*) the stop codon immediately following the *Hin*dIII site limits the length of the *lac*Z gene product to 68 amino acids.

The *Hin*dIII cleavage site of pBU10 was used for the insertion of various transcriptional terminators; only the results obtained with the major terminator of the bacteriophage *fd* genome, yielding the pLBU1 plasmid (Fig. 1), are described. Insertion of this terminator upstream from the *tet* gene resulted in a 90% reduction in the level of Tc resistance but no detectable change in the level of β-Gal activity in M15 deletion strains (Table 1). Seven independent plasmid isolates from such clones contained the expected 352-bp *Hin*dIII-generated DNA fragment carrying the *fd* terminator. Electrophoretic analysis of all seven *Bam*HI-cleaved isolates and DNA sequence analysis of one of these showed that in all instances the *fd* terminator had been inserted in an orientation opposite to the direction of transcription within the *fd* phage genome (Fig. 2; ref. 18). Sequence analysis also revealed a translational stop codon in frame with the α protein less than 10 bp downstream from the *Hin*dIII cleavage site at the junction between the *lac*-derived segment and the *fd*-derived DNA fragment (Fig. 2); the construct would be expected to produce a 71-amino acid α fragment.

For the cloning of exogenous promoters, the *lac* promoter in the *lac*/*tet* construct had to be removed or destroyed in such

Table 1. Properties specified by plasmid constructs

Plasmid	*E. coli* strain	Tc resistance, μg/ml	β-Gal activity Op.-Ind	β-Gal activity α-Compl.
pBU10	C600	20	+	
	M15	20		+
	M15^{iq}	<2		−
	+IPTG*	20		+
pBU12	C600	3	+	
	M15	3		−
pLBU1	C600	2	+	
	M15	2		+
pLBU3	C600	<1	+	
	M15	<1		−
pGBU207	C600	>70	+	
	M15	>70		+++
pBR322	C600	60	−	
	M15	60		−

The properties of transformed cells were determined by replica plating freshly grown cultures (logarithmic phase) at identical densities in microtiter dishes. Tc-containing plates were freshly prepared. Incubation was for 14 hr at 37°C. Isogeneic strains before and after transformation with pBR322 served as controls. Presence of the *lac* operator (Op.-Ind.) was determined in a *lac*$^+$ background. α-Protein synthesis (α-Compl.) was assessed in M15 deletion strains by evaluating the colony color on plates containing bromochloroindolyl galactoside.
* Isopropyl thiogalactoside.

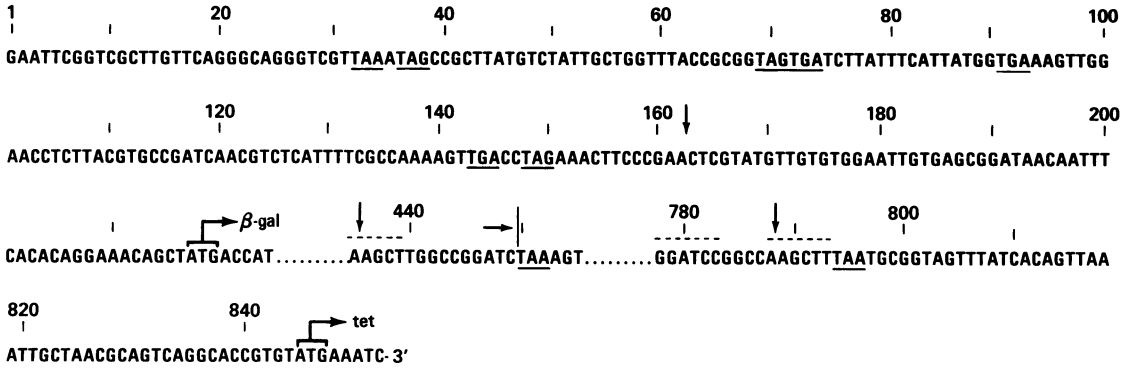

FIG. 2. Nucleotide sequences connecting the various cloned regions of pLBU3. Most leftward is the sequence of unknown origin intervening between the *Eco*RI site (position 1) and the −15 position of the *lac* promoter (163), followed by the sequence of the *lac* fragment (163–435) and the *fd* DNA (446–778); the last portion of sequence shows the transition between the *fd* DNA and the *tet* region (778–800). Stop codons in all three frames are underlined, and translation initiation sites of both the α fragment and the first *tet* protein are indicated. ----, *Eco*RI (1), *Hin*dIII (435, 778), and *Bam*HI (778) cleavage sequences; arrows, borders between the sequences of different origins.

a way that a site for the subsequent insertion of promoters was retained. To do this, we used the *Hpa* II cleavage site at position −17 of *lac* (5); a 250-bp fragment was isolated from a *Hpa* II/*Hin*dIII double digest of pBU10 and introduced into plasmid pBR322 to generate an *Eco*RI endonuclease cleavage site. Colonies that showed both a reduced level of Tc resistance and the presence of a *lac* operator sequence, which could be detected on the multicopy plasmid by its ability to bind the *lac* repressor and induce chromosomal β-Gal synthesis, were identified. Endonuclease analysis (*Hin*dIII/*Eco*RI double digest, data not shown) of plasmids recovered from several isolates yielded two types of vectors; one of these (represented by pBU12) harbored the expected 253-bp *lac* fragment, whereas the other (represented by pBU12a) yielded a 420-bp fragment.

DNA sequence analysis (Fig. 2) showed that in pBU12a a 160-bp fragment of unknown origin had been inserted between the *Eco*RI site and position −15 of the *lac* promoter; fortuitously, the fragment was found to contain two to three stop codons in each of the possible translational reading frames (Fig. 2), making the fragment an efficient terminator of any translation originating upstream from the translation initiation site for the α fragment. pBU12a was therefore used for our further experiments. Replacing the *Hin*dIII/*Sal* I segment of pBU12a (Fig. 1) with an identically generated fragment of pLBU1 carrying the *fd* terminator yielded pLBU3 (Fig. 1). Although this plasmid contained the *tet* region and a DNA sequence encoding the α fragment of *lac*, it conferred neither Tc resistance nor β-Gal activity to an *E. coli* M15 strain (Table 1) and thus seemed to be a candidate for the intended T5 promoter cloning vehicle.

Properties of Constructed Vectors. Expression of both the α-complementing peptide and the Tc resistance encoded by plasmid pBU10 is regulated by the *lac* promoter (Table 1); expression of the Lac phenotype and Tc resistance occur in strain C600 as a result of titration of the *lac* repressor by the operator located on the multicopy plasmid; such induction also results in expression of the *tet* gene carried by the plasmid, indicating *lac* promoter control of Tc resistance. The Lac phenotype occurs in the M15 mutant, which has part of the *lacZ* gene deleted from the chromosome, by α complementation. In the M15 *lac*I^q host, which overproduces the *lac* repressor, both *lac* expression and Tc resistance are repressed. Control of transcription into both gene segments by the *lac* promoter is further shown by the inducibility of *lac* expression by isopropyl thiogalactoside in the M15 *lac*I^q strain, and the concurrent loss of expression as a consequence of destruction of the *lac* promoter in the pBU12 plasmid (Table 1).

Interruption of the *lac* promoter sequence at a *Hpa* II site as described above abolishes the remaining transcription through the *fd* terminator into the Tc gene as well as α complementation; the residual Tc resistance encoded by pBU12a results from a partially restored promoter sequence at the *Eco*RI site, which is located within the −10 region of the *lac* promoter. This residual promoter activity is repressed by *lac* repressor [i.e., in the *lac*I^q strain (unpublished data)].

Cloning of Promoters of Coliphage T5. A population of about 200 short fragments of T5 DNA was obtained by double digestion of the 120-kilobase phage genome with *Hae* III and *Alu* I endonucleases. These fragments were ligated with excess synthetic *Eco*RI linkers, and the resulting molecules were cleaved with *Eco*RI endonuclease and ligated into the *Eco*RI cleavage site of pLBU3. Transformation of *E. coli* C 600 and selection for both β-Gal activity and high-level Tc resistance yielded 35 colonies resistant to Tc concentrations between 8 and 70 μg/ml. Plasmids were isolated from 13 clones resistant to 70 μg/ml, which appeared from earlier experiments using multicopy plasmids to be the highest level detectable in *E. coli* K-12 (21). Digestion of the various isolates with *Eco*RI liberated between 1 and 10 DNA fragments from each constructed plasmid. Complexing of such fragment mixtures with RNA polymerase, followed by filter binding analysis, identified between one and three fragments of each plasmid that interacted efficiently with the enzyme (Fig. 3); these fragments were isolated from polyacrylamide gels and individually recloned in pLBU3. In each case, they gave rise to colonies resistant to Tc at 70 μg/ml. Plasmids isolated from such clones carried the expected DNA fragments, as shown by *Eco*RI cleavage and gel electrophoresis. The promoter library obtained in this way contains about 25 different strong promoters of coliphage T5 (23).

Characterization of the pGBU207. One of the above plasmids (pGBU207, Fig. 4) that contained an *Eco*RI-generated fragment of only 212 bp was selected for further study; the efficiency RNA polymerase binding to the 212-bp fragment was compared with binding to two fragments carrying the previously characterized T5 promoters P_{25} and P_{26} (25). The results (Fig. 3) show that the putative promoter (P_{207}) present on the pGBU207 plasmid exhibits binding similar to that of P_{25} and P_{26}, which are among the most efficient *E. coli* RNA polymerase-binding sequences identified from any source (26).

The site(s) of *in vitro* transcription occurring on the pGBU207 plasmid were mapped by analysis of RNA transcripts made on plasmid DNA fragments produced by cleavage with different restriction endonucleases (Fig. 4). Cutting of the plasmid with

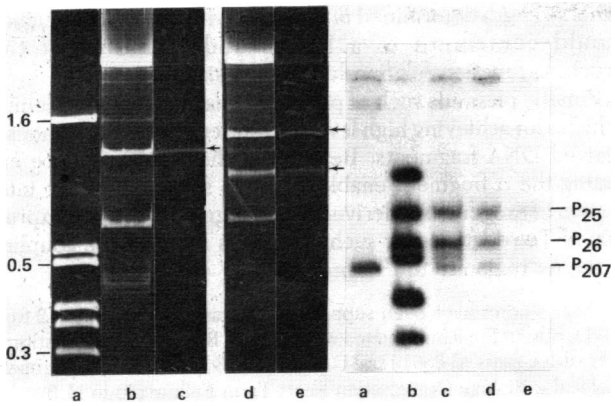

FIG. 3. Interaction of cloned coliphage T5 DNA fragments with *E. coli* RNA polymerase. (*Left*) Two examples from the library of T5 promoters. Lanes b and c are digests of pGBU34 and pGBU12 with *Eco*RI. In lanes c and e the fragments carrying promoters are identified (arrow) after complexing of the *Eco*RI digest with limiting concentrations of RNA polymerase followed by nitrocellulose filter binding. Such fragments were subsequently isolated and recloned. Lane a shows size standards (in kilobases). (*Right*) Nitrocellulose filter binding assay of a fragment mixture containing the strong T5 promoters P_{25} and P_{26} [*Hpa* II digest of *Hin*dIII-cleaved T5 DNA (22)] together with the 212-bp fragment isolated from pGBU207. Lanes e, d, and c show that the extent of polymerase binding at limiting but increasing amounts of the enzyme is the same for all the fragments (fragment sizes 400, 310, and 212 bp, respectively). Lanes a and b show the control gel for end-labeled fragments used to prepare the mixture. Analysis of the filter binding assays was on 10% acrylamide gels.

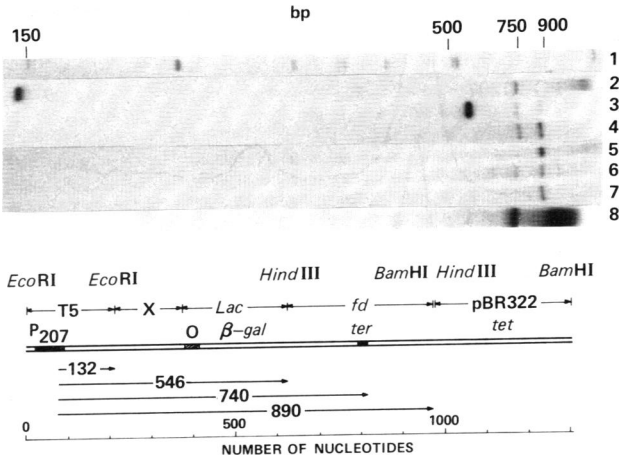

FIG. 4. Analysis of *in vitro* transcripts of pGBU207. (*Upper*) Electrophoretic separation of transcripts obtained under various conditions (lane 1, molecular weight standard; lanes 2–4, template cleaved with *Eco*RI, *Hin*dIII, and *Bam*HI, respectively). The transcripts of 130, 540, and 900 nucleotides, and the 740-nucleotide RNA terminated by the phage *fd* signal are by far the most abundant products of the plasmids transcription. Lanes 5–7 show transcription of *Bam*HI-digested pGBU207 at 50 mM KCl (5), at 50 mM KCl with *E. coli rho* protein, and at 150 mM KCl with GTP replaced by ITP (24). As shown by the disappearance of the 740-nucleotide RNA species, low salt and ITP abolish the termination. At 50 mM KCl, however, addition of *rho* protein restores termination to about 50% efficiency. Lane 8 shows transcripts of the covalently closed circular form of the plasmid at 150 mM KCl. (*Lower*) Overview of the transcriptional unit. X, 160-bp fragment integrated between the *Eco*RI and the *lac* DNA; arrows, sizes of transcripts expected from templates cleaved with various restriction endonucleases.

*Eco*RI endonuclease yielded primarily a single RNA species about 130 nucleotides long. The size of the transcript increased to 550 nucleotides when a *Hin*dIII digest of pGBU207 was used as a template. *Bam*HI-digested DNA yielded transcripts about 740 and 900 nucleotides long. Correlation of transcript length with the distance of the DNA cleavage site from a fixed point indicates that, in all of these instances, *in vitro* transcription was being initiated at the same promoter and that it progressed toward the *tet* region of the plasmid (Fig. 4). These experiments also show the functioning of the termination signal introduced between the *lac* fragment and the *tet* gene; under the high-salt conditions used for this *in vitro* transcription experiment, about 50% of the transcripts are terminated within the *fd* DNA fragment giving rise to the 740-nucleotide RNA, whereas read-through transcripts extend to the *Bam*HI cleavage site located 890 bp downstream from the promoter. The data in lanes 5–7 of Fig. 4 show that low ionic strength and replacement of GTP by ITP abolishes termination at position 740; on the other hand, addition of termination factor *rho* increases the termination efficiency at 50 mM KCl 20- to 50-fold.

To quantitate the intensity of transcription initiated at the P_{207} promoter relative to transcription of other regions of the pGBU207 plasmid, transcripts of whole plasmid DNA were hybridized to endonuclease fragments of the plasmid (Fig. 5). *In vivo* and *in vitro* transcriptions from the P_{207} promoter were 8 and 15 times, respectively, the transcription initiated at the *bla* promoter, which in turn was about 3 times more frequent than transcription of the *tet* region on the pBR322 (control) plasmid. Transcription from P_{207} into the *tet* region was prevented by the cloned terminator with almost 100% efficiency *in vitro* and about 93% efficiency *in vivo*.

DISCUSSION

The plasmid constructs described here permit the analysis of different transcription signals within the same genetic environment. By appropriately arranging their integration sites relative to two independent indicator functions (i.e., the α fragment of *E. coli* β-Gal and the *tet* region of pBR322), the interactions of promoters and terminators can be studied. Placement of stop codons between indicator genes uncouples their translation and allows use of their easily assayable products to estimate the relative efficiency of transcription initiation and termination signals. Because the adventitious 160-bp sequence (X in Fig. 4) preceding the coding region of the first indicator gene (the α peptide) contains stop codons in all three reading frames, any translation initiated within the cloned promoter-carrying upstream fragment is interrupted. This organization of translation signals not only permits reproducible synthesis of functional α and *tet* protein from translation start sites located within a polycistronic mRNA but most likely also maintains transcriptional polarity at a constant level.

Bacterial clones that express the products of the two indicator genes are identified by assaying for different levels of Tc resistance or screening for *lac* gene expression, thus enabling the cloning of strong terminators as well as promoters. Among the highly active terminators found in this way was the major terminator of bacteriophage *fd*, surprisingly integrated in a direction opposite to its natural orientation. This signal showed about a 93% efficiency of termination *in vivo* and nearly 100% efficiency *in vitro* (Fig. 5), although a major component of the natural termination signal [an oligo(dT) stretch downstream from the region of dyad symmetry of the terminator] is in a nonfunctional position when the terminator is integrated in the reverse orientation. However, the reverse orientation yields multiple stop codons between the *lac*-derived fragment and the termination signal (17), providing conditions for *rho*-dependent termination (27). Consequently, *rho* protein increases termination frequency *in vitro* 25- to 50-fold (Fig. 4, lane 6). The fact

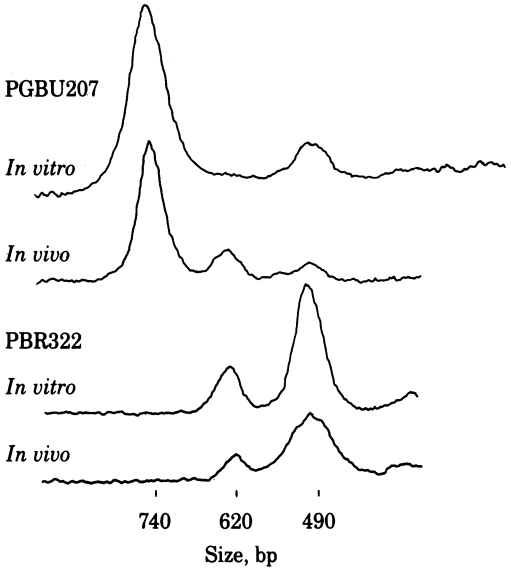

FIG. 5. Comparison of relative amounts of RNA produced under the control of P_{207}, P_{bla}, and P_{tet} *in vitro* and *in vivo*. ^{32}P-Labeled RNA made *in vitro* with pGBU207 and pBR322 as templates and *in vivo* in plasmid carrying C600 cells were hybridized to Southern blots to which the following DNA fragments were fixed: 740-bp *Eco*RI/*Bam*HI fragment of pGBU207 containing a 600-bp region under control of P_{207} (Fig. 4); 620-bp *Hin*dIII/*Sal* I; and 490-bp *Eco*RI/*Hin*cII fragment (the last two both were from pBR322 and contained a part of the *tet* and *bla* regions, respectively). The densitometric tracings of the autoradiograms show the relative amounts of ^{32}P-labeled RNA hybridizing to the DNA fragments indicated. The filter-bound RNA·DNA hybrids were extensively treated with RNase prior to autoradiography. The pGBU207-derived RNA hybridizing to the 620-bp fragment is the read-through product of P_{207} into the *tet* region.

that the cloned *fd* sequence terminates transcription efficiently despite the loss of the oligo(dT) stretch may be attributed to the newly acquired *rho* function which is not found for the termination in the *fd* genome (22).

Our plasmid constructs have permitted cloning of promoter-carrying fragments from the "early" region of bacteriophage T5, by using selection for read-through past the termination signal into the *tet* region—which results in colonies that are resistant to high levels of Tc. All such clones contain plasmids carrying at least one T5 DNA segment that binds efficiently to RNA polymerase (Fig. 3; ref. 23). We have not been able to stabilize such promoter-carrying fragments in any vector system lacking an efficient downstream termination signal. In some cases, plasmids expressing high-level Tc resistance had a reduced copy number. This effect was not observed when a Cm resistance gene was used in place of Tc.

The promoter-containing fragment cloned in pGBU207 binds RNA polymerase with high efficiency (Fig. 3) and initiates transcription toward the *tet* region, conferring a level of Tc resistance (70 μg/ml) that is 35 times higher than is seen when transcription is initiated from the *lac* promoter under comparable conditions. This finding is consistent with our analysis of transcripts produced *in vitro* from various regions of pGBU207 plasmid; the P_{207} promoter yields 15 times the RNA produced by the β-lactamase promoter, which in turn is about twice as efficient as the fully induced *lac* wild-type promoter (unpublished data).

The system described here allows the quantitative study *in vivo* of transcription initiation and termination signals having a wide range of efficiencies. Comparison of *in vivo* results with signal strength determined *in vitro* and with the DNA sequence should contribute to a better understanding of the structure–function relationship of such signals.

Finally, plasmids such as pGBU207 may be useful as cloning vehicles for achieving high levels of transcription of exogenously derived DNA fragments. Removal of the DNA sequence encoding the α fragment enables positive selection for the integration of exogenously derived DNA fragments because expression of Tc resistance by such constructs requires the coupling of the *tet* region to the T5 promoter.

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