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Improved Vector System for Constructing Transcriptional Fusions That Ensures Independent Translation of *lacZ*

THOMAS LINN* AND REBECCA ST. PIERRE

Department of Microbiology and Immunology, Faculty of Medicine, University of Western Ontario, London, Ontario N6A 5C1, Canada

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An improved vector system has been developed for the in vitro construction of transcriptional fusions to *lacZ*. The principal feature is an RNaseIII cleavage site inserted between the polylinker cloning site and the promoterless *lacZ* gene. When these vectors are used to construct transcriptional fusions, the subsequent cleavage of the hybrid mRNA at the RNaseIII site generates an unchanging 5′ end for the *lacZ* mRNA. In contrast to earlier vectors, this feature helps to ensure independent translation of the *lacZ* mRNA and, thus, the level of β-galactosidase produced should accurately reflect the frequency of transcription of the upstream DNA sequences. Additional modifications of the vectors include removal of a weak transcriptional terminator between the cloning site and *lacZ*, insertion of a terminator downstream of *lac*, and alteration of restriction endonuclease cleavage sites to facilitate the in vitro construction of fusions. Both multicopy plasmid (pTL61T) and single-copy lambda (λTL61) vectors have been assembled. These vectors should be generally useful in scanning for transcriptional regulatory signals.

Gene fusions have become important tools for the analysis of gene regulation in both procaryotic and eucaryotic systems. In general, the attachment of the regulatory sites of a given gene upstream of a reporter gene that encodes an easily assayed enzyme facilitates the analysis of expression of the affixed gene. The reporter gene may be used in either transcriptional (operon) fusions, where it retains its own translational start site but is dependent on the attached DNA for transcription, or in translational (protein) fusions, where both its transcription and translation are dependent on signals in the attached upstream DNA. The history and a wide variety of specific applications of gene fusions have been reviewed by Silhavy and Beckwith (45).

Although a variety of reporter genes have been used in the construction of gene fusions, the most common is *lacZ* of *Escherichia coli*. The product, β-galactosidase, is stable in many cellular backgrounds and can be precisely quantitated by a very simple and sensitive colorimetric assay. Moreover, the construction of fusions to *lacZ* extends all the experimental convenience of the *lac* genetic system to any given gene. That is, by using the available *lac* technology, one can readily screen and select for altered levels of β-galactosidase produced from the gene fusion. This greatly facilitates the regulatory analysis of the upstream attached gene.

Numerous vector systems have been described for the construction of gene fusions to *lacZ* (45). Both multicopy (plasmid) and single-copy (bacteriophage) vectors have been used to construct gene fusions by either in vivo or in vitro manipulations. We previously described a vector system for the in vitro construction of transcriptional fusions to *lacZ* which had some advantages over the then currently available vectors (26). Both plasmid (pTL25) and phage (λTL25) versions were assembled, but we have used primarily the single-copy vector.

With extensive use of λTL25, we identified several aspects of the vector which required modification. These modifications included (i) removal of a weak transcriptional terminator between the cloning site and *lacZ*, (ii) insertion of a strong terminator downstream of the *lac* sequence, (iii) alteration of restriction endonuclease cleavage sites in the polylinker cloning site and elsewhere in the vector, and most importantly, (iv) addition of an RNaseIII processing site between the cloning site and *lacZ*. This last feature allows cleavage of the hybrid mRNA, providing a consistent 5′ end for the *lacZ* mRNA, which in turn helps ensure independent translation of *lacZ* irrespective of what sequences are fused upstream. The improved *lacZ* transcriptional fusion vector does not suffer from the artifacts seen with previous vectors and is superior when used in scanning for transcriptional regulatory sites. We have used this vector to examine the composite *rplJL rpoBC* operon, which encodes both the L10 (*rplJ*) and L7/12 (*rplL*) ribosomal proteins and the β (*rpoB*) and β′ (*rpoC*) subunits of RNA polymerase. The transcription profile produced is consistent with the known regulatory sites present in the operon.

MATERIALS AND METHODS

Media. Cultures for β-galactosidase determinations were routinely grown in AB4 medium (AB base medium [11] supplemented with 0.4% glucose and 1% Casamino Acids). For phage lysates, bacteria were grown at 37°C on agarose plates (BBL Microbiology Systems, Cockeysville, Md.) (10 g Trypticase, 5 g NaCl, 10 g agarose per liter). Bacteria transformed with recombinant plasmids were selected on plates containing LB agar (30) supplemented with 20 μg of ampicillin per ml.

Recombinant DNA. The standard recombinant DNA methods used were essentially as described by Maniatis et al. (28). Restriction endonucleases, DNA modifying enzymes, and linkers were obtained from either Boehringer Mannheim Canada Ltd. (Dorval, Quebec, Canada) or Pharmacia Canada Inc. (Baie d'Urfe, Quebec, Canada). Custom oligonucleotides were synthesized by the Regional DNA Synthesis Laboratory (Calgary, Alberta, Canada).

DNA fragments and λ vector arms generated by restriction endonuclease digestion were resolved by electrophoresis on agarose gels. The appropriate bands were excised from the gels, and the DNA was extracted by using the

* Corresponding author.

Geneclean protocol (Bio 101 Inc., La Jolla, Calif.). Recovered fragments were ligated in vitro with T4 DNA ligase. Plasmid recombinants were recovered in TL11 (26), a Δ(*argF-lacIOPZYA*)*205* derivative of HB101, by using the frozen storage III transformation protocol of Hanahan (18). Lambda recombinants were recovered by in vitro packaging (28) of the ligation mixture and plating onto a lawn of C600 (3). Plugs were picked from single plaques with Pasteur pipettes, and the phage was suspended in 1 ml of SM buffer (28). Plate lysates were made essentially as described by Maniatis et al. (28), except 50 to 100 μl of the phage suspension was mixed with 0.3 ml of *E. coli* C600 and the mixture was plated on agarose plates (BBL). Lysis was generally complete within 6 to 8 h, and the titer was routinely 2×10^9 to 5×10^9 PFU/ml. This stock lysate was used both for DNA isolation and lysogen formation.

The use of gel-purified DNA fragments for the in vitro constructions greatly increased the efficiency of production of the desired recombinants, and this in turn decreased the effort required for screening and recovery of such recombinants. In most cases the only construct that could result from the direct ligation of the purified DNA fragments was the recombinant of interest. However, in all cases the structures of the recovered plasmid and λ recombinants were confirmed by extensive restriction endonuclease analysis. Plasmid DNA was prepared for screening by using the boiling method of Holmes and Quigley (20). Recombinant λ DNA was prepared for screening by using the method of Davis et al. (13) or Maniatis et al. (28). Both procedures were followed by a spermine precipitation (21) of the λ DNA.

Lysogen formation and β-galactosidase assays. To isolate lysogens, MG4 (26), a *recA* Δ(*argF-lacIPOZYA*)*205* derivative of MG1655 (17), was infected at a multiplicity of approximately 0.1 with a recombinant phage as follows. A fresh overnight culture of MG4 grown in YT medium (30) was centrifuged, and the cell pellet was suspended in an equal volume of SM buffer (28) containing 10 mM $MgSO_4$. A 0.9-ml portion of the suspended cells was mixed with 0.1 ml of the plate lysate stock of the desired recombinant phage, and the mixture was incubated at 32°C for 20 min to allow phage adsorption. Appropriate dilutions (generally 0.1 ml of 10^{-4} and 10^{-5} dilutions) were spread on either MacConkey lactose or LB plus X-Gal plates (30), depending on whether the phage produced high or low levels, respectively, of β-galactosidase. Lysogens were identified by colony color after incubation at 37°C.

The low multiplicity of infection generates primarily monolysogens. However, to be certain the lysogens carried a single copy of the recombinant phage, the prophage copy number was always checked by the Ter excision test (33): 2 ml of the lysogen (grown to ca. 2×10^8 cells per ml) in AB4 or LB was brought to 10 mM $MgSO_4$, infected with 40 μl (multiplicity of infection = 2) of the heteroimmune phage λ762 (*imm*$^{\lambda}$ Δ*cI*) (35) and grown at 37°C on a tube rotator. After 2 h, 3 drops of chloroform were added and incubation was continued for 10 min to lyse the culture. Dilutions of the lysate were spotted onto a freshly inoculated lawn of *E. coli* 159 (*imm*$^{\lambda}$ *cI ind* lysogen) (39) to determine the resulting titer of the recombinant *imm*^{21} phage. With MG4, the *recA* host used for lysogen formation, monolysogens produce between 10^3 and 10^4 PFU/ml, whereas multiple lysogens yield a titer ca. 10^3-fold higher.

Monolysogens of the recombinant phage were grown at 37°C with shaking in AB4 medium. Samples were taken at culture densities of about 0.1 and 0.2 optical density at 600 nm. β-galactosidase was assayed as described by Miller (30) by using chloroform and sodium dodecyl sulfate to permeabilize the cells.

Construction of *lacZ* transcriptional fusion vectors. The basic steps in the construction of pTL38, pTL40, pTL61T, λTL39, and λTL61 are described in Results and Discussion. Segments of pTL25 (26), pFR109 (44), pKK161-8 (8), pKK233-2 (2), pRS415 (46), and pAR3435 (43) were used in the construction of pTL38 and pTL61T. Segments of λL47.1 (27) and TL25 (26) were used in the construction of λTL39.

The complete sequence of pTL61T (GenBank accession no. M29896) can be deduced from our work and published sources. pTL61T (8,565 base pairs [bp], numbered from the start of the *Eco*RI site in the polylinker): 1 to 238 = nucleotides 1 to 238 shown in Fig. 1c (this region includes the polylinker, T7 RNaseIII processing site, and the remaining segment of the *trp* sequence to the W205 deletion junction), 239 to 3392 = *lac* sequences (10, 19, 22), *lacZ* extends from 256 to 3327, and the remaining segment of *lacY* extends from 3382 to 3392 (this version of *lacZ* carries an uncharacterized mutation [6] removing the *Eco*RI site present near the 3′ end of the wild-type *lacZ* gene); 3393 to 3890 = 932 to 1428 of pBR322 (37, 47); 3891 to 4886 = two tandem copies of the *rrnB* transcriptional terminator region, 6416 to 6913 of Brosius et al. (9) (this region was inserted from pKK161-8) (8); 4887 to 7826 = 1426 to 4363 of pBR322 (37, 47) plus TT, except that the *Pst*I site of pBR322 (CTGCAG) has been altered to CTAGAC (the region containing this change was inserted from pKK233-2) (2); 7827 to 8565 = 11722 to 12460 of pRS551 (46) (this region contains four tandem copies of the *rrnB*T1 terminator).

Most of the sequence of λTL61 can be deduced from our work and published sources. λTL61 is approximately 45 kilobases (kb) in length. Starting from the left end of the genome, base pairs 1 to 19,399 are from λL47.1 (27), which is identical to wild-type lambda (12), except the usual *Bam*HI site at 5505 has been removed by an uncharacterized mutation. The next 8,243 bp come from the transcriptional fusion plasmids. The segment is inserted in the opposite orientation with respect to the left arm of the lambda genome such that 19400 to 19420 = 1446 to 1426 of pBR322 (37, 47); 19421 to 20416 = two tandem copies of the *rrnB* terminator region, 6913 to 6416 of Brosius et al. (9); 20417 to 21194 = 1428 to 652 of pBR322 (37, 47); 21195 to 27404 = *lac* region, with 22336 to 22944 = *lacA* (19), 23011 to 24261 = *lacY* (10), and 24316 to 27387 = *lacZ* (22); 27405 to 27642 = 238 to 1 of the *trp*-T7 RNaseIII site-polylinker shown in Fig. 1. The lambda sequences then resume with 27643 to 35918 of the vector = 26104 to 34379 of lambda (12). The next ca. 2 kb of DNA is the *imm*^{21} substitution from phage 21 (25). Although the endpoints of the substitution are known in the lambda sequence (12) and the approximate length of the region is 2 kb, the exact sequence is not known. For purposes of numbering, we have set the size of this region at 2,000 bp, therefore, 35919 to 37918 = *imm*^{21}. Next, 37919 to 39803 = 38617 to 40501 of lambda (12). Finally, 39804 to 45011 = 43307 to 48514 of lambda (12). The lambda DNA between these two segments is missing as the result of the *nin5* deletion.

The pTL61T and λTL61 vectors along with the complete sequence of pTL61T and details of construction are available upon request to T.L.

Construction of *rplrpo-lacZ* transcriptional fusions. Segments of the *rplJLrpoBC* gene cluster were used to construct transcriptional fusions in either the λTL39 or λTL61 vectors. The source of the *rplJLrpoB* DNA was either λ*drif*^{d}18 (24) or

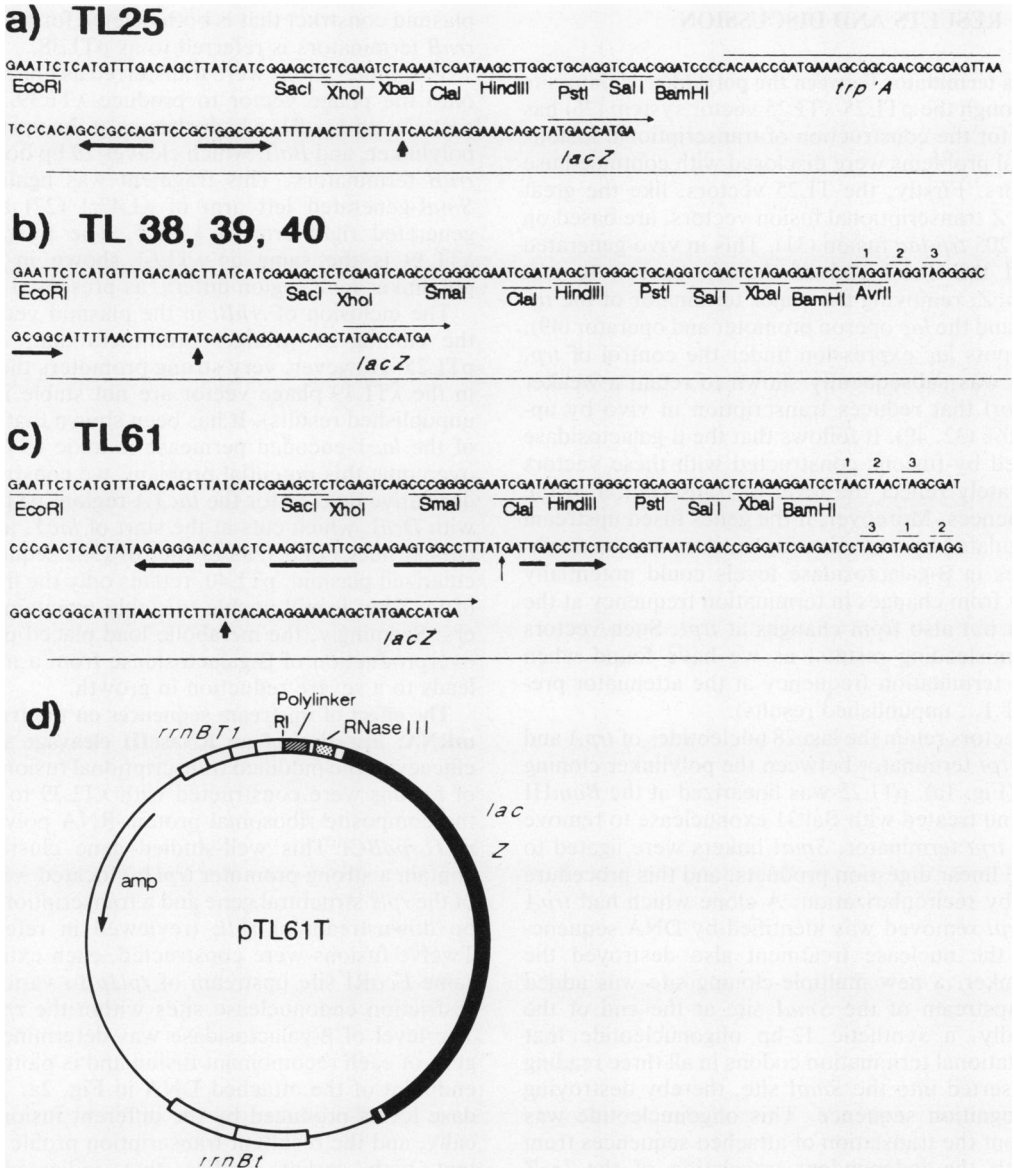

FIG. 1. *lacZ* transcriptional fusion vectors. (a through c) The DNA sequence of the polyrestriction site linkers in the *lacZ* transcriptional fusion vectors. The restriction endonuclease sites are underlined and labeled. The start of *lacZ* is overlined. Translational termination codons resulting from the insertion of oligonucleotides are overlined and numbered with respect to the relative reading frame. The thick vertical arrow indicates the junction produced from the W205 *trp-lac* deletion. (a) TL25. The overlined *trp'A* region indicates the remaining 3' segment of *trpA*. The thick underlining designates the inverted repeat sequence that forms the stem of *trpt*. (b) TL38, TL39, and TL40. The thick underlining indicates the remaining nonfunctional segment of the *trpt* stem. (c) TL61. The thick underlining designates the inverted repeat sequence that forms the RNaseIII processing site. The actual cleavage site is designated by the thin vertical arrow (14). The 5' end of the processed *lacZ* mRNA will be the G residue to the right of the arrow. (d) Structure of pTL61T. The thin lines designate sequences from pBR322. The sequence of the polylinker-RNaseIII region is shown in panel c. RI indicates the *Eco*RI site of the polylinker. The open boxes labeled *rrnBt* designate two tandem copies of the *rrnB* transcriptional terminator region, each of which contains two terminators, *T1* and *T2*. The open boxes labeled *rrnBT1* indicate four tandem copies of the *rrnBT1* terminator. The exact size and sequence of each region of the plasmid is described in Materials and Methods.

a plasmid derivative carrying this specific region (pRJ1; R. Jones and T. Linn, unpublished results). The fragments inserted into the fusion vectors all extended from the same *Eco*RI site 1,070 bp upstream of *rplJp* to the various restriction sites listed below. These fragments were isolated and ligated between the left and right arms of the lambda vectors, and the recombinants were recovered by the standard procedures described above. The exact endpoint of the *rplJL-rpoB*-specific DNA in each fusion and the restriction endonuclease used to generate the terminus are as follows: (1) 1728, *Dra*I; (2) 1990, *Sma*I; (3) 2159, *Hin*dIII; (4) 2250, *Sna*BI; (5) 2449, *Eco*RI; (6) 2676, *Dde*I; (7) 2732, *Nar*I; (8) 2813, *Aat*II; (9) 2878, *Xmn*I; (10) 2895, *Hin*cIII; (11) 2946, *Sal*I; (11a) 3095, *Xho*II; and (12) 3373, *Kpn*I. The number of each fusion corresponds to those presented in Fig. 2. The nucleotide numbering is according to Post et al. (38). The sites in the polylinker to which these fragments were joined are as follows: (1) *Sma*I, (2) *Sma*I, (3) *Hin*dIII, (4) *Sma*I, (5) *Eco*RI, (6) *Sma*I, (7) *Sma*I, (8) *Sma*I, (9) *Sma*I, (10) *Sma*I, (11) *Sal*I, (11a) *Eco*RI, and (12) *Xho*I.

RESULTS AND DISCUSSION

Removal of a terminator between the polylinker cloning site and *lacZ*. Although the pTL25-λTL25 vector system (26) has proven useful for the construction of transcriptional fusions to *lacZ*, several problems were disclosed with continued use of these vectors. Firstly, the TL25 vectors, like the great majority of *lacZ* transcriptional fusion vectors, are based on the original W205 *trp-lac* fusion (31). This in vivo-generated deletion fused the distal end of the *trp* operon to just upstream of *lacZ*, removing the major terminator of the *trp* operon (*trpt'*) and the *lac* operon promoter and operator (49). This deletion puts *lac* expression under the control of *trp*, but the fusion was subsequently shown to retain a weaker terminator (*trpt*) that reduces transcription in vivo by approximately 40% (32, 49). It follows that the β-galactosidase levels produced by fusions constructed with these vectors will not accurately reflect the level of transcription of the upstream sequences. Moreover, if the genes fused upstream of *lacZ* are regulated by a termination-antitermination mechanism, changes in β-galactosidase levels could potentially result not only from changes in termination frequency at the site of interest but also from changes at *trpt*. Such vectors can produce misleading results, as we have found when examining the termination frequency at the attenuator preceding *rpoB* (T.L., unpublished results).

The TL25 vectors retain the last 28 nucleotides of *trpA* and the complete *trpt* terminator between the polylinker cloning site and *lacZ* (Fig. 1a). pTL25 was linearized at the *Bam*HI site (Fig. 1a) and treated with Bal 31 exonuclease to remove the functional *trpt* terminator. *Sma*I linkers were ligated to the ends of the linear digestion products, and this procedure was followed by recircularization. A clone which had *trpA* and most of *trpt* removed was identified by DNA sequencing. Because the nuclease treatment also destroyed the original polylinker, a new multiple-cloning site was added immediately upstream of the *Sma*I site at the end of the deletion. Finally, a synthetic 12-bp oligonucleotide that contains translational termination codons in all three reading frames was inserted into the *Sma*I site, thereby destroying the *Sma*I recognition sequence. This oligonucleotide was added to prevent the translation of attached sequences from interfering with the independent translation of the *lacZ* sequence. The nucleotide sequence of the resulting polylinker-W205 *trp-lac* region is shown in Fig. 1b. Fusions constructed with the *trpt*-deleted vector produced an average of twofold more β-galactosidase than the analogous fusions constructed with the TL25 vectors.

Insertion of transcriptional terminators downstream of *lacZ*. The second problem we encountered with the TL25 vectors was the difficulty of cloning very strong promoters, with the plasmid vector being much more sensitive to promoter strength than the phage vector (T.L., unpublished results). Others have reported the inability to clone strong promoters in given plasmid vectors, presumably as a result of a high level of transcription continuing through the plasmid origin of replication, which apparently interferes with normal plasmid replication and copy number control (1, 16, 48). To insulate the plasmid sequences from transcription initiated by a strong promoter, tandem copies of the *rrnB* terminator region, each containing both T1 and T2, were placed downstream of *lacZYA*. These terminators, inserted at the unique *Ava*I site of the pBR322 sequences in the fusion vector, were transferred from pKK161-8 (8) by using flanking restriction enzyme cleavage sites. The intermediate plasmid construct that is both deleted for *trpt* and carries the *rrnB* terminators is referred to as pTL38.

These alterations were transferred as one segment of DNA onto the phage vector to produce λTL39. pTL38 was digested with *Eco*RI, which cleaves at the upstream end of the polylinker, and *Bal*I, which cleaves 19 bp downstream of the *rrnB* terminators. This fragment was ligated between the *Sma*I-generated left arm of λL47.1 (27) and the *Eco*RI-generated right arm of λTL25. The overall structure of λTL39 is the same as λTL61 shown in Fig. 3, but its polylinker-*lacZ* region differs, as presented in Fig. 1.

The inclusion of *rrnBt* in the plasmid vector has allowed the cloning of stronger promoters than was possible in pTL25. However, very strong promoters that can be cloned in the λTL39 phage vector are not stable in pTL38 (T.L., unpublished results). It has been shown that overproduction of the *lacY*-encoded permease is toxic to the cell (36). To overcome this potential problem, we constructed a plasmid derivative deleted for the *lacYA* region. pTL38 was cleaved with *Dra*I, which cuts at the start of *lacY*, and *Nae*I, which cuts at nucleotide 1283 of the pBR322 sequence. The recircularized plasmid, pTL40, retains only the first three codons of *lacY* but is still unable to stably maintain strong promoters. Seemingly, the metabolic load placed on cells by gross overproduction of β-galactosidase from a multicopy vector leads to a severe reduction in growth.

The effect of upstream sequences on the translation of *lacZ* mRNA: insertion of an RNaseIII cleavage site. To test the efficacy of the modified transcriptional fusion vector, a series of fusions were constructed with λTL39 to various sites in the composite ribosomal protein-RNA polymerase operon, *rplJLrpoBC*. This well-studied gene cluster is known to contain a strong promoter (*rplJp*) located ≈370 bp upstream of the *rplJ* structural gene and a transcriptional attenuator 69 bp downstream of *rplL* (reviewed in references 14, 40). Twelve fusions were constructed, each extending from the same *Eco*RI site upstream of *rplJp* to various downstream restriction endonuclease sites within the *rplJLrpoB* genes. The level of β-galactosidase was determined in monolysogens of each recombinant fusion and is plotted versus the 3' endpoint of the attached DNA in Fig. 2a. The β-galactosidase levels produced by the different fusions varied erratically, and the resultant transcription profile was not consistent with earlier studies that indicated the level of transcription through *rplJL* is uniform, followed by a decrease in the no. 6 to no. 7 interval as a result of termination at a transcriptional attenuator (4, 14, 41).

It is generally assumed in the construction of transcriptional fusions that the overall translational efficiency of the reporter gene sequence from the hybrid mRNA is independent of the attached upstream sequences and, therefore, the level of β-galactosidase produced should directly reflect the level of transcription of the attached gene. However, in the assembly of such fusions, the mRNA sequences immediately upstream of *lacZ* will change with each construct. These changing sequences could potentially alter: (i) the stability of the hybrid mRNA (5) and/or (ii) the translational initiation frequency of *lacZ*. In such cases the level of β-galactosidase produced will not be solely dependent on the frequency of transcription.

Alterations in translational initiation may result from changes in the secondary structure of the hybrid mRNA in the vicinity of the *lacZ* ribosome binding site and translational initiation codon. Such changes could make the initiation region more or less accessible. Indeed, Munson et al. (34) have shown that point mutations which altered the

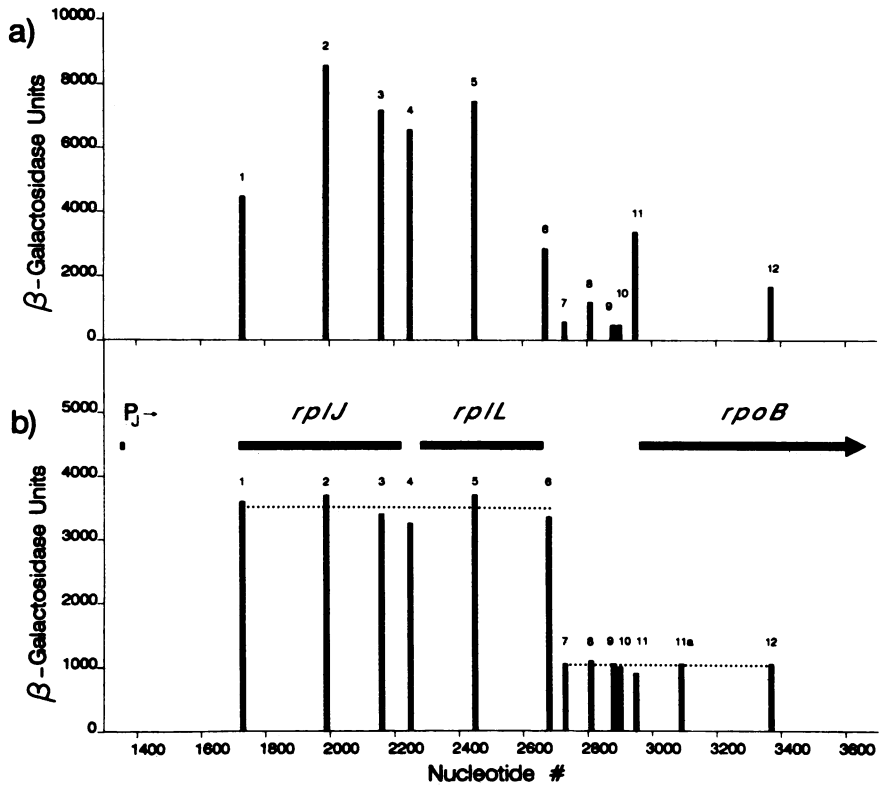

FIG. 2. Transcription profile of the *rplJL-rpoB* region. DNA fragments extending from a site upstream of *rplJp* to the endpoints indicated by the bars were used to construct transcriptional fusions to *lacZ* in either λTL39 (a) or λTL61 (b). The level of β-galactosidase produced by monolysogens of each recombinant fusion is plotted. Each β-galactosidase level represents the average of four to eight determinations, with a standard deviation of less than 5%. The nucleotide numbering is according to Post et al. (38). The position of *rplJp* (P_J), *rplJ*, *rplL*, and the 5′ end of *rpoB* are shown. The exact endpoint of the *rplJL-rpoB*-specific DNA in each fusion is described in Materials and Methods.

secondary structure in the region including the *lacZ* ribosome-binding site resulted in a sixfold variation in the level of β-galactosidase expression. Some of the results with our *lacZ* transcriptional fusions are consistent with this finding. Specifically, 1,200 U of β-galactosidase was produced when a DNA fragment including *rplJp* and extending to the no. 11 endpoint was fused to the *Sal*I site upstream of *lacZ* in the polylinker of λTL39 (Fig. 2a). However, the β-galactosidase activity was increased to 3,250 U by modifying the fusion with the removal of only the 6-bp *Xba*I site from the polylinker (Fig. 1b). This effect is dependent upon the DNA attached upstream of the *Sal*I site, since the β-galactosidase levels produced by the fusion of several other DNA fragments did not vary with the presence or absence of the *Xba*I site (data not shown).

Computer analysis of the RNA sequences from the two fusions suggested that the construct containing the *Xba*I site forms a stable secondary structure which sequesters the *lacZ* ribosome-binding site, whereas in the construct lacking the *Xba*I site, the *lacZ* translational initiation region would be free (data not shown). These results suggested that the variation in the β-galactosidase levels seen during operon scanning may be due to alterations in the secondary structure of the mRNA, which in turn affect the translational efficiency of the *lacZ* sequence.

In an attempt to overcome the problem of changing secondary structures in the vicinity of the *lacZ* translational initiation site, an RNaseIII processing site (23, 42) was introduced between the polylinker and *lacZ* on the transcriptional fusion vectors. Thus, in an RNaseIII-proficient strain, the nascent hybrid mRNA will be cleaved at the RNaseIII site to generate a constant 5′ end on the *lacZ* mRNA. The RNaseIII processing site used was the R1.1 site from the T7 bacteriophage genome (15). pTL40 was cleaved at the *Bam*HI and *Avr*II sites in the polylinker (Fig. 1b), and a 93-bp *Bam*HI-*Bgl*II-generated fragment from pAR3435 (43) that carries the R1.1 RNaseIII site was inserted. Secondly, a synthetic oligonucleotide carrying translational termination codons in all three reading frames was introduced between the polylinker and the RNaseIII cleavage site by insertion at the *Bam*HI site. This should prevent ribosomes originating in the translation of the upstream sequences from reading through into the RNaseIII site and disrupting the formation of the secondary structure required for cleavage. The DNA sequence of the polylinker-RNaseIII site-*lacZ* N-terminal region was confirmed by the dideoxy method and is presented in Fig. 1c. This plasmid was further modified by the insertion of four tandem copies of the *rrnBT1* transcriptional terminator upstream of the *Eco*RI site (9). This was accomplished by replacement of the DNA sequence from the *Sca*I site in the *amp* gene to the *Eco*RI site in the polylinker with the corresponding fragment of pRS415 which includes the terminators (46). The terminators should block transcription from upstream plasmid promoters. The structure of the resulting plasmid, pTL61T, is shown in Fig. 1.

The region of pTL61T containing the RNaseIII cleavage site was then transferred from the plasmid onto the lambda transcriptional fusion vector. The plasmid was digested with *Xba*I, which cleaves in the polylinker, and *Sac*I, which cleaves within *lacZ*. The *Xba*I-*Sac*I-generated fragment was

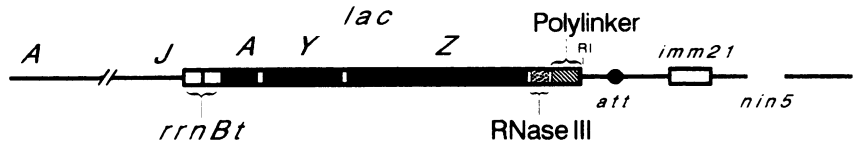

FIG. 3. Structure of λTL61. The thin line to the left designates the left arm of the lambda genome and is derived from λL47.1 (27). The thin line to the right of the *Eco*RI site (RI) of the polylinker designates the right arm of the lambda genome, including a functional attachment site (*att*), the phage 21 immunity region substitution (imm^{21}) and the *nin5* deletion. This right arm, although directly donated by λTL25, was originally derived from λNM616 (29). The sequence of the polylinker-RNaseIII region is presented in Fig. 1c. The vector contains the complete *lacZYA* genes, followed by two tandem copies of the *rrnBt* transcriptional terminator region. The drawing is not to scale. The size and sequence of each region is described in Materials and Methods.

then ligated between the homologous sites of digested λTL39 to produce λTL61 (Fig. 3). The background β-galactosidase levels produced by the vectors alone are 600 U for pTL61T and 71 U for monolysogens of λTL61. The level of β-galactosidase produced by the λTL61 vector allows one to easily identify promoter-containing recombinants on MacConkey lactose plates. Monolysogens of the vector are very light pink, while recombinants producing more than 200 U of β-galactosidase are red. Most of the residual β-galactosidase production by the vector appears to result from a low level of initiation within the polylinker, as removal of the sequences between the *Eco*RI and *Bam*HI sites reduced the background level of the plasmid to 146 U.

The same set of fusions of *rplJLrpoB* sequences to *lacZ* described earlier with the λTL39 vector were reconstructed by using λTL61. The β-galactosidase levels produced in monolysogens of the transcriptional fusions are plotted according to the position of the fusion endpoint in Fig. 2b. There is a striking contrast in the transcription profile as compared with the results in which λTL39 was used. The six fusions ending within the *rplJ* and *rplL* structural genes all produce a consistent level of β-galactosidase averaging around 3,500 U. The β-galactosidase level then drops in the 56-bp interval between the fusion no. 6 and fusion no. 7 endpoints, the region known to contain a transcriptional attenuator. Finally, seven different fusion endpoints downstream of no. 7 and extending to approximately 400 bp into *rpoB* give consistent β-galactosidase levels averaging around 1,000 U.

Thus, the inclusion of an RNaseIII cleavage site between the polylinker and *lacZ* appears to have remedied the aberrant variations seen in the transcription profile produced with λTL39. Presumably this is due to the production of an unvarying 5′ end for the *lacZ* mRNA as a result of RNaseIII cleavage. Consequently, the translational initiation frequency and stability of the *lacZ* mRNA should remain constant irrespective of what sequences were originally attached upstream. If the upstream sequences were to in some way interfere with formation of the secondary structure required for RNaseIII cleavage (e.g., formation of an alternative secondary structure including part of the RNaseIII site), it is possible that the β-galactosidase level would not accurately reflect the frequency of transcription, but to date we have found no examples of this occurring. Therefore, a vector carrying such a cleavage site appears to meet the requirements of a bona fide transcriptional fusion vector.

Alteration of the available cloning sites. During the construction of pTL61T and λTL61, restriction endonuclease cleavage sites in the polylinker and elsewhere in the vectors were modified to facilitate the in vitro assembly of transcriptional fusions. The ten restriction endonuclease cleavage sites present in the polylinker of pTL61T and λTL61 are indicated in Fig. 1c. Eight of these sites are unique in pTL61T (Table 1), so DNA fragments generated by any of these enzymes, either singly or in combination, can be directly ligated into the vector. The use of pairwise combinations of these enzymes allows convenient directional

TABLE 1. Cloning sites in *lacZ* transcription fusion vectors

Vector	Restriction endonucleases		
	Sites unique to polylinker	Generating left arm of lambda genome, including *lac* from λTL61	Generating right arm of lambda genome including *att*
pTL61T	*Eco*RI, *Xho*I, *Hin*dIII, *Pst*I (*Nsi*I, *Hgi*AI),[a] *Sma*I (blunt termini), *Sal*I, *Xba*I (*Spe*I, *Avr*II, *Nhe*I), *Bam*HI (*Bcl*I, *Bgl*II, *Mbo*I, *Sau*3A, *Xho*II)		
λTL61		*Eco*RI, *Xho*I, *Sma*I (blunt termini), *Hin*dIII, *Sal*I, *Xba*I (*Spe*I, AvrII, *Nhe*I), *Bam*HI (*Bcl*I, *Bgl*II, *Mbo*I, *Sau*3A, *Xho*II)	*Eco*RI, *Sac*I, *Xba*I (*Spe*I, *Avr*II, *Nhe*I)
λNM540[b]			*Hin*dIII
λKV4[c]			*Kpn*I
λRS205[d]			*Sal*I (*Xho*I)

[a] Restriction endonucleases listed in parentheses generate compatible termini.
[b] Borck et al. (7).
[c] G. Ralling and T. Linn, unpublished observations.
[d] Bertrand et al. (6).

cloning. Moreover, because the polylinker contains a *Sma*I site, DNA fragments produced by any enzyme that generates blunt termini can be ligated to this site. In addition, three of the sites (*Pst*I, *Xba*I, and *Bam*HI) produce termini that are compatible to those produced by a number of other restriction endonucleases (Table 1). Therefore, a wide variety of restriction endonucleases can be used to generate DNA fragments that can be directly inserted into the plasmid vector without further manipulation of the termini.

In λTL61, the *Xba*I and *Eco*RI sites are unique in the vector, so DNA fragments produced by these enzymes can be inserted directly. Seven of the restriction endonuclease cleavage sites in the polylinker are unique to the left arm of the phage (Table 1). That is, the complete left arm of the vector, including the *lac* region, can be generated by cleavage with any of these enzymes. Therefore, promoter-containing DNA fragments with termini downstream of the direction of transcription that are compatible with any of these sites can be directly ligated to the complete left arm of the phage vector. Digestion of λTL61 with either *Eco*RI, *Xba*I, or *Sac*I will produce the intact right arm of the vector genome, including the *att* site necessary for the formation of stable lysogens. Accordingly, any enzyme that leaves termini compatible with these can be used to generate the upstream end of promoter-containing DNA fragments. The production of directly useable upstream termini is not limited to these enzymes, since one can use the right arm of the lambda genome derived from other commonly used cloning vectors. The only requirements are that the restriction endonuclease site used is unique in the right arm of the genome and that the arm carries the functional *att*, *int*, and *c*I genes necessary for the formation of stable lysogens. Several of the phage vectors we have frequently used to supply right arms are listed in Table 1. By using this collection of vectors, DNA fragments produced by a large assortment of restriction enzymes can be used directly to construct single-copy transcriptional fusions. To reduce the effort required in recovering the desired recombinant fusion, we routinely use agarose gel electrophoresis to purify not only the DNA fragments to be inserted but also the appropriate left and right vector arms produced by restriction endonuclease digestion. Since the size of the λTL61 genome is 45 kb and the maximum amount of DNA that can be packaged into virions is approximately 51 kb, the vector should accommodate DNA fragments up to 6 kb in size.

We strongly recommend using the single-copy vector for the construction of transcriptional fusions. Fusions carried on multicopy plasmids are subject to several intrinsic problems. Plasmid copy number can vary with the size of the DNA insert and the strength of the cloned promoter (1). Certain complete or truncated gene products can be detrimental to the cell when expressed at high levels from multicopy plasmids. This provides a selective pressure for reduced expression of the gene or reduction in the plasmid copy number. Also, multiple copies of some genes or overexpression of their products could potentially titrate regulatory components present in low numbers in the cell and thus lead to abnormal expression. All of these problems can result in alterations of the β-galactosidase level, which then no longer accurately reflect the normal level of transcription of the test sequences. Although there are some instances in which fusions on multicopy vectors are useful, we have used the single-copy vector exclusively for the critical analysis of transcriptional regulation. The main utility of the plasmid vector may be in the two-step construction of fusions with DNA fragments that cannot be directly ligated into the phage vector. For example, a *Sma*I-*Pst*I fragment can first be inserted between the homologous sites on the plasmid and then subsequently removed by using the flanking *Eco*RI and *Xba*I sites for insertion into the phage vector. Alternatively, fusions initially made in the plasmid vector could be transferred onto lambda via in vivo recombination by making use of the phage vectors developed by Simons et al. (46).

Overall, the λTL61 and pTL61T vectors should provide a generally useful system for the construction of transcriptional fusions to *lacZ*, which will allow accurate scanning for transcriptional regulatory sites.

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LITERATURE CITED

1. **Adams, L. W., and G. W. Hatfield.** 1984. Effects of promoter strengths and growth conditions on copy number of transcription-fusion vectors. J. Biol. Chem. **259:**7399–7403.
2. **Amann, E., and J. Brosius.** 1985. 'ATG vectors' for regulated high-level expression of cloned genes in *Escherichia coli*. Gene **40:**183–190.
3. **Appleyard, R. K.** 1954. Segregation of new lysogenic types during growth of a doubly lysogenic strain derived from *Escherichia coli* K12. Genetics **39:**440–452.
4. **Barry, G., C. Squires, and C. L. Squires.** 1980. Attenuation and processing of RNA from the *rplJL-rpoBC* transcription unit of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **77:**3331–3335.
5. **Belasco, J. G., G. Nilsson, A. von Gabain, and S. N. Cohen.** 1986. The stability of *E. coli* gene transcripts is dependent on determinants localized to specific mRNA segments. Cell **46:** 245–251.
6. **Bertrand, K. P., K. Postle, L. V. Wray, Jr., and W. S. Reznikoff.** 1984. Construction of a single-copy promoter vector and its use in analysis of regulation of the transposon Tn*10* tetracycline resistance determinant. J. Bacteriol. **158:**910–919.
7. **Borck, K., J. D. Beggs, W. J. Brammer, A. S. Hopkins, and N. E. Murray.** 1976. The construction *in vitro* of transducing derivatives of phage lambda. Mol. Gen. Genet. **146:**199–207.
8. **Brosius, J.** 1984. Plasmid vectors for the selection of promoters. Gene **27:**151–160.
9. **Brosius, J., T. J. Dull, D. D. Sleater, and H. F. Noller.** 1981. Gene organization and primary structure of a ribosomal RNA operon from *Escherichia coli*. J. Mol. Biol. **148:**107–127.
10. **Buchel, D. E., B. Gronnelborn, and B. Müller-Hill.** 1980. Sequence of the lactose permease gene. Nature (London) **283:** 541–545.
11. **Clarke, D. J., and O. Maaloe.** 1967. DNA replication and the division cycle in *Escherichia coli*. J. Mol. Biol. **23:**99–112.
12. **Daniels, D. L., J. L. Schroeder, W. Szybalski, F. Sanger, A. R. Coulson, G. F. Hong, D. F. Hill, G. B. Peterson, and F. R. Blattner.** 1983. Complete annotated lambda sequence, p. 519–676. *In* R. W. Hendrix, J. W. Roberts, F. W. Stahl, and R. A. Weisberg (ed.), Lambda II. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. **Davis, R. W., D. Botstein, and J. R. Roth.** 1980. Advanced bacterial genetics, p. 109. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
14. **Downing, W. L., and P. P. Dennis.** 1987. Transcription products from the *rplKAJL-rpoBC* gene cluster. J. Mol. Biol. **194:**609–620.
15. **Dunn, J. J., and W. F. Studier.** 1983. Complete nucleotide sequence of bacteriophage T7 DNA and the locations of T7 genetic elements. J. Mol. Biol. **166:**477–535.
16. **Gentz, R., A. Langer, A. C. Y. Chang, S. N. Cohen, and H. Bujard.** 1981. Cloning and analysis of strong promoters is made possible by the downstream placement of a RNA termination signal. Proc. Natl. Acad. Sci. USA **78:**4936–4940.

17. **Guyer, M. S., R. R. Reed, J. A. Steitz, and K. B. Low.** 1981. Identification of a sex-factor-affinity site in *E. coli* as αδ. Cold Spring Harbor Symp. Quant. Biol. **45**:135–140.
18. **Hanahan, D.** 1985. Techniques for transformation of *E. coli*, p. 109–135. *In* D. M. Glober (ed.), DNA cloning, vol. 1. IRL Press, Washington, D.C.
19. **Hediger, M. A., D. F. Johnson, D. P. Nierlick, and I. Zabin.** 1985. DNA sequence of the lactose operon: the *lacA* gene and the transcriptional termination region. Proc. Natl. Acad. Sci. USA **82**:6414–6418.
20. **Holmes, D. S., and M. Quigley.** 1981. A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem. **114**: 193–197.
21. **Hoopes, B. C., and W. R. McClure.** 1981. Studies on the selectivity of DNA precipitation by spermine. Nucleic Acids Res. **9**:5493–5504.
22. **Kalnins, H., K. Otto, U. Ruther, and B. Müller-Hill.** 1983. Sequence of the *lacZ* gene of *Escherichia coli*. EMBO J. **2**:593–597.
23. **King, T. C., R. Sirdeshmukh, and D. Schlessinger.** 1986. Nucleolytic processing of ribonucleic acid transcripts in procaryotes. Microbiol. Rev. **50**:428–451.
24. **Kirschbaum, J. B., and E. Konrad.** 1973. Isolation of a specialized transducing bacteriophage carrying the beta subunit gene for *Escherichia coli* ribonucleic acid polymerase. J. Bacteriol. **116**:517–526.
25. **Liedke-Kulke, M., and A. D. Kaiser.** 1967. The c-region of coliphage 21. Virology **32**:475–481.
26. **Linn, T., and G. Ralling.** 1985. A versatile multiple- and single-copy vector system for the *in vitro* construction of transcriptional fusions to *lacZ*. Plasmid **14**:134–142.
27. **Loenen, W. A. M., and W. J. Brammar.** 1980. A bacteriophage vector for cloning large DNA fragments made with several restriction enzymes. Gene **10**:249–257.
28. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
29. **Mileham, A. J., H. R. Revel, and N. E. Murray.** 1980. Molecular cloning of the T4 genome; organization and expression of the *frd*-DNA ligase region. Mol. Gen. Genet. **179**:227–239.
30. **Miller, J. H.** 1972. Experiments in molecular genetics. p. 352. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
31. **Mitchell, D., W. Reznikoff, and J. Beckwith.** 1975. Genetic fusions defining *trp* and *lac* operon regulatory elements. J. Mol. Biol. **93**:331–350.
32. **Mott, J. E., J. L. Galloway, and T. Platt.** 1985. Maturation of *Escherichia coli* tryptophan operon mRNA: evidence for 3′ exonucleolytic processing after rho-dependent termination. EMBO J. **4**:1887–1891.
33. **Mousset, S., and R. Thomas.** 1969. Ter, a function which generates the ends of the mature lambda chromosome. Nature (London) **221**:242–244.
34. **Munson, L. M., G. D. Stromo, R. L. Niece, and W. Reznikoff.** 1984. *lacZ* translational initiation mutations. J. Mol. Biol. **177**: 663–683.
35. **Murray, N. E., W. J. Brammar, and K. Murray.** 1977. Lambdoid phages that simplify the recovery of *in vitro* recombinants. Mol. Gen. Genet. **150**:53–61.
36. **Padan, E., T. Arbel, A. Rimon, A. B. Shira, and A. Cohen.** 1983. Biosynthesis of the lactose permease in *Escherichia coli* minicells and effect of carrier amplification on cell physiology. J. Biol. Chem. **258**:5666–5673.
37. **Peden, K. W. C.** 1983. Revised sequence of the tetracycline-resistance gene of pBR322. Gene **22**:277–280.
38. **Post, L. E., G. D. Strycharz, M. Nomura, H. Lewis, and P. P. Dennis.** 1979. Nucleotide sequence of the ribosomal protein gene cluster adjacent to the gene for RNA polymerase subunit β in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **76**:1697–1701.
39. **Ptashne, M.** 1967. Isolation of the λ phage repressor. Proc. Natl. Acad. Sci. USA **57**:306–313.
40. **Ralling, G., and T. Linn.** 1984. Relative activities of the transcriptional regulatory sites in the *rplKAJLrpoBC* gene cluster of *Escherichia coli*. J. Bacteriol. **158**:279–285.
41. **Ralling, G., and T. Linn.** 1987. Evidence that Rho and NusA are involved in termination in the *rplL-rpoB* intercistronic region. J. Bacteriol. **169**:2277–2280.
42. **Robertson, H. D.** 1982. *Escherichia coli* ribonuclease III cleavage sites. Cell **30**:669–672.
43. **Rosenberg, A. H., B. N. Lade, D. Chui, S. Lin, J. J. Dunn, and F. W. Studier.** 1987. Vectors for selective expression of cloned DNAs by T7 RNA polymerase. Gene **56**:125–135.
44. **Shapira, S. K., J. Chou, F. V. Richaud, and M. J. Casadaban.** 1983. New versatile plasmid vectors for expression of hybrid proteins coded by a cloned gene fused to *lacZ* gene sequences encoding an enzymatically active carboxyl-terminal portion of β-galactosidase. Gene **25**:71–82.
45. **Silhavy, T. J., and J. R. Beckwith.** 1985. Uses of *lac* fusions for the study of biological problems. Microbiol. Rev. **49**:398–418.
46. **Simons, R. W., F. Houman, and N. Kleckner.** 1987. Improved single and multicopy *lac*-based cloning vectors for protein and operon fusions. Gene **53**:85–96.
47. **Sutcliffe, J. G.** 1979. Complete nucleotide sequence of the *Escherichia coli* plasmid pBR322. Cold Spring Harbor Symp. Quant. Biol. **43**:77–90.
48. **Wong, E. M., M. A. Muesing, and B. Polisky.** 1982. Temperature-sensitive copy number mutants of ColE1 are located in an untranslated region of the plasmid genome. Proc. Natl. Acad. Sci. USA **79**:3570–3574.
49. **Xian-Ming, Y., L. M. Munson, and W. S. Reznikoff.** 1984. Molecular cloning and sequence analysis of *trp-lac* fusion deletions. J. Mol. Biol. **172**:355–362.