Multiple and single copy "-lac-based fusion vectors designed for use with strong promoters

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Introduction

Genetic fusions have been widely used for studying gene expression and regulation in both procaryotes and eucaryotes (Silhavy and Beckwith, 1985; Lis et al., 1983; Price et al., 1987; Mansour et al., 1990). Transcriptional, or operon, fusions, where a promoter of interest is fused to a reporter gene, can be used to measure the relative strength of a promoter and regulation occurring at the transcriptional level. Translational, or protein, fusions can detect regulation at the translational level and can also provide information regarding the translation of a gene of interest. For translational fusions, the promoter and N-terminal portion of a gene of interest is fused in-frame to a reporter gene. Because the *E. coli* lacZ gene product, $\beta$-galactosidase ($\beta$-gal) is relatively insensitive to heterologous protein sequences at its N-terminus, it is the most commonly used reporter gene. We have used lacZ fusions extensively in our studies on IS10 transposase (tnp) expression and autoregulation of the rnc operon (Simons and Kleckner, 1983; Case et al., 1988, 1989, 1990; Matsunaga and Simons, in preparation). However, because we have found difficulty constructing fusions to genes with very strong promoters using our lacZ fusion vectors (Simons et al., 1987), we sought an alternative method for constructing lacZ fusions.

Early complementation experiments on the lacZ gene divided the gene into three regions, $\alpha$, $\beta$, and $\gamma$ (for reviews, see Ullmann and Perrin, 1970, and Zabin and Villarejo, 1975). The $\alpha$ portion corresponds to the operator-proximal fourth, the $\beta$ portion corresponds to the center, and the $\gamma$ portion corresponds to the terminal third of the gene. Functional $\beta$-galactosidase expression can be achieved when the protein is expressed intact from the lacZ gene, or when a lacZ gene containing a mutation at least one-fourth of the distance into the gene is complemented by defective $\beta$-galactosidase (M15 protein) expressed from the strain, M15, which has a deletion in the operator-proximal portion of the lacZ gene (Ullmann et al., 1967). This is referred to as $\alpha$-complementation. Because the $\alpha$-complemented enzyme is stable in growing cells (Villarejo et al., 1972), we reasoned that it should be possible to construct fusions to the $\beta$ fragment of $\beta$-galactosidase that can be complemented by the M15 protein. Since mutationally altered proteins may be rapidly degraded in bacteria (Goldberg and Dice, 1974; Goldberg and St. John, 1976), these fusions might be expected to result in lower $\beta$-gal levels than fusions to an intact lacZ gene. Therefore, such $\alpha$-complementing fusion vectors might be useful for constructing genetic fusions to genes with strong promoters.

Experimental and Discussion

Description of monocopy plasmid vectors for constructing $\alpha$-complementing lacZ fusions

We designed a set of vectors analogous to our original lacZ fusion vectors (Simons et al., 1987). The structure of the vectors is shown in Figure 1 and their essential features are summarized in Table I. Their constructions are briefly diagrammed in Figure 2. The plasmids contain the following elements (clockwise): *ori* and *bla* from pBR322 (Sutcliffe, 1979) for selecting ApR transformants; T14 transcriptional terminators to block upstream transcription (Brosius et al., 1981; Simons et al., 1987); unique cloning sites (*Eco*RI, *Sma*I and *Bam*HI) for constructing fusions; *lacZ* lacking both transcriptional and translational starts (pRS1551 and pRS1552) or only its transcriptional start (pRS1553 and pRS1554); and finally, a single T1 transcriptional terminator to prevent transcription of the fusion from interfering with plasmid functions.

Transferring multicopy fusions to single copy prophages

It would be useful to generate an intact lacZ gene for experimental purposes since $\alpha$-complementing fusions might be expected to exhibit unreliable $\beta$-galactosidase expression due to degradation of the truncated lacZ gene product. Fusions constructed with the multicopy $\alpha$-complementation vectors can be transferred to a specific F' phage by genetic recombination in *vivo*, and the recombinant phages integrated as single prophages in the host chromosome. Homologous recombination occurs at sequences flanking the insert, *bla* upstream and *lac* downstream, resulting in Ap$^3$, Lac$^+$ recombinant phages that can be isolated from blue plaques (Figure 3).
Construction of a fusion between the strong tetD promoter and the ρ'-complementing fragment
In our studies on the regulation of the tetD and tetC genes (Schollmeier and Hillen, 1984) from the unique loop region of Tn10 (Figure 4A), we were unable to construct transcriptional or translational fusions between tetD and an intact lacZ gene in the absence of the tetC gene, which we now know encodes a repressor of tetD and tetC expression (Chapter IV). Presumably, the lac genes were being overexpressed to levels in which the cells could not tolerate. If this were true, we reasoned that it might be possible to construct tetD-ρ'-fusions if these fusions display lower 0-galactosidase expression. We cloned a fragment of DNA containing the tetD promoter (pD) and N-terminal sequences into transcriptional and translational ρ'-complementing fusion vectors and transformed these constructs into DH5ρ' and JM101 (these strains express the M15 protein) and plated on X-gal. Blue transformants were found to contain the correct insert. These fusions were subsequently transferred to prophages by homologous recombination with RS468 then lysogenized into single copy in the chromosome of DR459. The structure of the pD-ρ'-transcriptional fusion is shown in Figure 4B. Since we were unable to construct multicopy fusions between tetD and an intact lacZ gene but we were able to construct tetD-ρ'-complementing fusions, these results demonstrate the utility of these ρ'-complementing vectors in constructing lacZ fusions with genes containing strong promoters.

Comparison of single copy fusions originally constructed by fusing inserts to an intact lacZ gene or an ρ'-complementing fragment of lacZ
Transcriptional fusions were constructed to the ρ'-complementing fragment of lacZ and the intact lacZ gene using several promoters: the pIN and pOUT promoters of IS10 (Simons et al., 1983) and the lacUV5 promoter. The fusions were transferred to prophages and subsequently lysogenized into single copy. 0-galactosidase expression from these fusions and the tetD fusions described above was measured (Table II and data not shown). The results in Table II demonstrate that in single copy, fusions originally constructed to the ρ'-complementing fragment of lacZ display essentially identical 0-galactosidase activities as those originally constructed to an intact lacZ gene. The ~3-fold difference seen for the pOUT fusions is probably due to triple versus single lysogenization of prophages carrying these fusions. Nevertheless, these ρ'-complementing vectors can be used for constructing lacZ fusions to many genes, from those that are weakly expressed to those that are very highly expressed since the promoters we examined express anywhere from ~20 to ~6000 units of 0-galactosidase activity. It should be emphasized that upon transfer to the 8 prophage, the ρ'-fusions were restored to intact lacZ fusions and these fusions exhibit the same level of 0-gal activity as fusions that were constructed to an intact lacZ gene from the start. Therefore, these ρ'-complementing fusions provide a method for constructing lacZ fusions to genes with strong promoters that avoids the construction of a multicopy fusion to an intact lacZ gene.

Experimental procedures
Strains, media and chemicals
Media, growth conditions and transformation procedures were as described (Simons et al., 1987). When used, supplements were added at the following concentrations: ampicillin, 150 μg/ml; X-gal, 40 μg/ml. Media components were purchased from Difco, and antibiotics from Sigma. Restriction enzymes and modifying enzymes were purchased from New England Biolabs. X-gal was purchased from 5-Prime to 3-Prime.

DNA manipulations
DNA preparation and manipulation and other routine in vitro recombinant procedures were as described (Maniatis et al., 1982).

Derivation of plasmid structures from pRS551 sequence
Starting with the sequence of pRS551 (which is available upon request), the sequence of the ρ'-complementing vectors can be derived as follows:
pRS551 (12,460 bp) is shown on the sequence and restriction maps.
pRS552 (12,340 bp) = pRS551 "deleted" for bp 17 to bp 136, inclusive.
pRS415 (10,752 bp) = pRS551 "deleted" for bp 10,014 to bp 11,721, inclusive.
pRS414 (10,632 bp) = pRS415 "deleted" for bp 17 to bp 136, inclusive.
pRS1274 (10,752 bp) = pRS415 with a substitution at the polylinker:
  replace:  \[\text{TTGGGGATCG GAATTCCGG GGATCCGGAC AACC---(415)}\]
  with:    \[\text{TTGGGGATCC CCGGGAATTC CGATCCGGAC AACC---(1274)}\]
pRS591 (10,632 bp) = pRS414 with a substitution at the polylinker:
  replace:  \[\text{TTGGGGATCG GAATTCCGG GGATCCCGTC GTTT---(414)}\]
  with:    \[\text{TTGGGGATCC CCGGGAATTC CGATCCCGTC GTTT---(591)}\]
pRS1348 (5404 bp) = pRS415 "deleted" for bp 952-6299, inclusive.
pRS1347 (5284 bp) = pRS414 "deleted" for bp 832-6179, inclusive.
pRS1377 (5404 bp) = pRS1274 "deleted" for bp 952-6299, inclusive.
pRS1376 (5284 bp) = pRS591 "deleted" for bp 832-6179, inclusive.
pRS1553 (5605 bp) = pRS1348 with an insertion starting at bp 952 and extending to bp 1157:
  \[\text{TCGACCAATTCCCAATTCC + T1 + GGAATTGGGGATCTCGAC}\]
  bp 1158-5605 = bp 6305-10752 of pRS415
pRS1554 (5605 bp) = same as pRS1553, except substitute pRS1377 for pRS1348 and pRS1274 for pRS415
pRS1551 (5485 bp) = pRS1347 with an insertion starting at bp 832 and extending to bp 1037:
  \[\text{TCGACCAATTCCCAATTCC + T1 + GGAATTGGGGATCTCGAC}\]
  bp 1038-5485 = bp 6185-10,632 of pRS414
pRS1552 (5485 bp) = same as pRS1551, except substitute pRS1376 for pRS1347 and pRS591 for pRS414

**Derived sequence near the Clal-Sall deletion junction**

pRS1553:
  bp 1-951 = bp 1-951 of pRS415
  bp 952-964 = TCGACCAATTCCC
  bp 965-1145 = AATTCC + T1 + GGAATT
  bp 1146-1157 = GGGGATCTCGAC
  bp 1158-5605 = bp 6305-10,752 of pRS415

pRS1554:
  same as pRS1553, except substitute pRS1274 for pRS415

pRS1551:
  bp 1-831 = bp 1-831 of pRS414
  bp 832-844 = TCGACCAATTCCC
  bp 845-1025 = AATTCC + T1 + GGAATT
  bp 1026-1037 = GGGGATCTCGAC
bp 1038-5485 = bp 6185-10,632 of pRS414

pRS1552: same as pRS1551 except substitute pRS591 for pRS414

References


Villarejo, M., Zamenhof, P. J., and Zabin, I. (1972) $\beta$-Galactosidase: in vivo $\beta$-complementation. J. Biol. Chem. 247:2212-

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Cloning sites</th>
<th>Plasmid size (bp)</th>
<th>Maximum insert for transfer to ( \mathbf{8} ) (kb)</th>
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<tr>
<td>pRS1551</td>
<td>EcoRI-SmaI-BamHI</td>
<td>5485</td>
<td>4.5</td>
</tr>
<tr>
<td>pRS1552</td>
<td>BamHI-SmaI-EcoRI</td>
<td>5485</td>
<td>4.5</td>
</tr>
<tr>
<td>pRS1553</td>
<td>EcoRI-SmaI-BamHI</td>
<td>5605</td>
<td>4.5</td>
</tr>
<tr>
<td>pRS1554</td>
<td>EcoRI-SmaI-BamHI</td>
<td>5605</td>
<td>4.5</td>
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</table>

\(^a\)See Figure 1.

\(^b\)Values are estimates of the maximum size of fragments that can be cloned into the plasmid vectors and subsequently crossed to \( \mathbf{8RS468} \) (see Figure 3).
Table II. Expression of transcriptional lacZ fusions

<table>
<thead>
<tr>
<th>Fusion plasmid or phagea</th>
<th>Fusion Number</th>
<th>Description</th>
<th>Expressionb</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>(A) Multicopy fusions</td>
<td></td>
</tr>
<tr>
<td>pRS475</td>
<td>5695</td>
<td>placUV5</td>
<td></td>
</tr>
<tr>
<td>pRS495</td>
<td>355</td>
<td>pIN</td>
<td></td>
</tr>
<tr>
<td>pRS586</td>
<td>4505</td>
<td>pOUT</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(B) Single copy fusions (from intact lacZ construction)</td>
<td></td>
</tr>
<tr>
<td>8RS74</td>
<td>1735</td>
<td>placUV5</td>
<td></td>
</tr>
<tr>
<td>8RS86</td>
<td>20</td>
<td>pIN</td>
<td></td>
</tr>
<tr>
<td>8RS235</td>
<td>170</td>
<td>pOUT</td>
<td></td>
</tr>
<tr>
<td>8RS417</td>
<td>6000</td>
<td>pD</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(C) Single copy fusions (from &quot;-fragment construction)</td>
<td></td>
</tr>
<tr>
<td>8RS481</td>
<td>1842</td>
<td>placUV5</td>
<td></td>
</tr>
<tr>
<td>8RS483</td>
<td>25</td>
<td>pIN</td>
<td></td>
</tr>
<tr>
<td>8RS485</td>
<td>55</td>
<td>pOUT</td>
<td></td>
</tr>
<tr>
<td>8RS640</td>
<td>5670</td>
<td>pD</td>
<td></td>
</tr>
</tbody>
</table>

apRS475 and pRS586 were described previously (Case et al., 1988). pRS495 contains the 556 bp AccI-BglII fragment containing the outer 336 bp of IS10-Right fused to lacZ in pRS415 (see Figure 4; Simons et al., 1987). Single copy fusions in (B) were obtained by crossing the multicopy fusions in (A) with 8RS45 (Simons et al., 1987). The pD single copy fusion was crossed with 8RS45 in the presence of a multicopy plasmid carrying the tetC gene. Single copy fusions in (C) were obtained by crossing "-complementing fusions with 8RS468.

b$\beta$-galactosidase assayed in DR459 according to Simons et al. (1987).
Figure 1. Multicopy $\beta$-complementing vectors
Structures and partial restriction maps are shown. All plasmids contain three unique cloning sites upstream from the $\beta$-complementing fragment of lacZ that are identical to those in our intact lacZ vectors (see Simons et al., 1987, for sequences at the cloning site). pRS1551 and pRS1552 are protein fusion vectors, pRS1553 and pRS1554 are operon fusion vectors. $\beta$, $\beta'$-fragment of lacZ; $\beta'$, $\beta'$-fragment lacking the first seven codons of lacZ; open boxes, genes or parts thereof, except for boxes with T1, which are transcriptional terminators (Brosius et al., 1981). Some unique restriction sites are shown.

Figure 2. Construction of $\beta$-complementing vectors
A) The plasmids shown in Figure 1, pRS1551, pRS1552, pRS1553, and pRS1554, were constructed by first deleting a 5351 bp ClaI-SalI fragment from pRS414, pRS591, pRS415, and pRS1274 (Simons et al., 1987), filling-in the overhangs and ligating, which restores the SalI site. This results in plasmids pRS1347, pRS1376, pRS1348, and pRS1377, respectively. A single T1 terminator (Brosius et al., 1981) was placed downstream of the SalI site by exchanging the PstI-SalI fragment from pRS1349 that contains the T1 terminator with the PstI-SalI fragment from pRS1347, pRS1376, pRS1348, and pRS1377, resulting in the $\beta$-fusion vectors pRS1551, pRS1552, pRS1553, and pRS1554, respectively (see Figure 1).

Figure 3. Transferring fusions from plasmid to phage
The strategy for plasmid-to-phage transfer is shown. A) Fusion vector pRS1553; B) pRS1553 containing an EcoRI-BamHI insert; C) phage 8RS468 necessary for transfer; D) the resulting recombinant phage from recombination between elements in B and C. 8RS468 is analogous to 8RS45 except that 8RS468 contains more N-terminal lacZ sequences than does 8RS45. It was constructed by making a BamHI-PvuII deletion of pRS415, then transferring this plasmid to phage by crossing with 8RS74 and screening for white plaques. All procedural details and descriptions of 8RS45, 8RS74 and pRS415 can be found in Simons et al. (1987). Boxes, genes or parts thereof; thin lines, plasmid backbone DNA; double lines, phage DNA; heavy line, putative recombination arms; bla, the proximal half of bla (bla is oriented right-to-left in all elements); lacZ', deletion of 5'-terminal sequences up to the PvuII site ~100 bp into the lacZ coding region. E=EcoRI, S=SmaI, B=BamHI, P=PvuII.

Figure 4. Structure of Tn10 and a pD-$\beta$-complementing fusion
A) The genetic structure of Tn10 is shown with respect to the hisG9424::Tn10 insertion (Foster et al., 1981). It contains two nearly-identical inverted repeat copies of insertion sequence IS10, IS10-Left and IS10-Right (Foster et al., 1981; Halling et al., 1982). The two IS10-Right promoters, pIN and pOUT, are shown. The four known genes located in the central region of Tn10 are shown, along with their directions of transcription. Relevant restriction sites are indicated and are numbered with respect to the outside end of IS10-Right, which is on the right-hand side of this page. B) An $\beta$-complementing fusion to the tetD promoter, pD, is shown. The 424 bp BglII-Nhel fragment was cloned into BamHI-EcoRI-digested pRS1554 after a BamHI linker was inserted at the BglII site and an EcoRI linker was inserted at the Nhel site. Open boxes, genes or parts thereof; hatched boxes, T1 transcriptional terminators (Brosius et al., 1981).