A DERIVATIVE OF PLASMID $\lambda dv$ WHOSE 
pRoR-tof IS REPLACED BY 
lacPO-lacI

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We have prepared plasmid pLIOP3, a derivative of $\lambda dv$ whose pRoR-tof region was replaced by lacPO (lactose promoter-operator)-lacI (repressor for the lac operon). pLIOP3 did not transform Escherichia coli polA. A mutant of pLIOP3 which could multiply as a plasmid was isolated after hydroxylamine mutagenesis. The mutant had two base substitutions in lacPO, which made the operator constitutive. The mutant could become transformed even after the removal of the replication origin of pBR322 which was contained in pLIOP3. The mutant which contains only the replication origin of $\lambda$ is named pLmI3. The synthesis of pLmI3 DNA was stimulated about 10-fold by the addition of an inducer of lactose promoter and the DNA recovered after induction had the same structure and biological activity as that recovered before induction. Cells carrying pLmI3 were killed by the induction. The replication of pLmI3 DNA depended on $O$ and $P$ gene products and the "ori" region of $\lambda dv$.

We have prepared several derivatives of $\lambda dv$ whose pRoR-tof region was replaced by lacPO (lactose promoter-operator). Some biological properties of these derivatives (pLOP1, 2 and 3) were examined and reported in a previous paper (1). One of these derivatives, pLOP3 was maintained in Escherichia coli when it carried F'lacI. The synthesis of pLOP3 DNA was strongly stimulated by the addition of isopropyl-$\beta$-D-thiogalactopyranoside (IPTG, an inducer of lactose promoter), most of the plasmid DNA was recovered from the membrane fraction and had little biological activity (1). The E. coli polA strain was not transformed by pLOP3. We thought that when lacI gene was inserted so that the lac-repressor was synthesized under the control of lacPO, the plasmid might multiply stably in polA mutant. A derivative of pLOP3 was constructed by inserting the lacI gene just behind lacPO to

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synthesize the repressor under the control of lacPO. This plasmid, pLIOP3, mimics αdv structurally more than pLOP3 does, for in αdv the tof gene, which is a repressor of pRoR, is present just behind the pRoR promoter (2). Though pLIOP3 did not transform E. coli polA, we have isolated a mutant (pLmI3) which can do so. In this paper, we will report some characters of the mutant.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this paper are listed in Table 1.

Construction of plasmid pLIOP3 and its derivatives. The structural gene of lacI whose promoter and terminator region was removed was isolated from pMPC1 (3). Appropriate adaptors prepared from the digests of pBR322 were ligated to this fragment to change the ends to BamHI. It was inserted at the BamHI site present just behind the lacPO of pLOP3 as shown in Fig. 1.

pLIZ3 is a derivative of pLIOP3 in which the structural gene of β-galactosidase (4) was inserted at the BglII site of the O gene (see Fig. 3). This plasmid produced an active β-galactosidase as a fused protein with O protein and was used

<table>
<thead>
<tr>
<th>Table 1. Bacterial strains and plasmids.</th>
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<tbody>
<tr>
<td><strong>Strain</strong></td>
</tr>
<tr>
<td><em>E. coli</em> strains</td>
</tr>
<tr>
<td>BMH408</td>
</tr>
<tr>
<td>WA802</td>
</tr>
<tr>
<td>WA802polA polA mutant of WA802</td>
</tr>
<tr>
<td>MC1000</td>
</tr>
<tr>
<td>HI1006</td>
</tr>
<tr>
<td>JM103</td>
</tr>
<tr>
<td>MM383</td>
</tr>
<tr>
<td>Plasmids</td>
</tr>
<tr>
<td>αdv021</td>
</tr>
<tr>
<td>pBR322</td>
</tr>
<tr>
<td>p344</td>
</tr>
<tr>
<td>pBR-αdv</td>
</tr>
<tr>
<td>pLOP3</td>
</tr>
<tr>
<td>pMPC1</td>
</tr>
<tr>
<td>pMC1403</td>
</tr>
<tr>
<td>p/Cm′</td>
</tr>
<tr>
<td>pTH12-6</td>
</tr>
<tr>
<td>pJS213-5</td>
</tr>
</tbody>
</table>

Abbreviations: Su−, nonsense suppressor; Δ, deletion; Ap′, ampicillin resistance; Cm′, chloramphenicol resistance; Km′, kanamycin resistance; Te′, tetracycline resistance.

The other genetic symbols are those used by Demerec et al. (25).
to assay the transcription of the lactose promoter.

**Mutagenesis of pLIOP3.** pLIOP3 was mutagenized by treating it with hydroxylamine hydrochloride following the method described by HASHIMOTO and SEKIGUCHI (5). pLIOP3 DNA (25 μg/ml) was incubated at 37°C for 20 hr in 0.4 M hydroxylamine solution at pH 6.0. Ampicillin-resistant transformants of *E. coli* WA802 polA were recovered with the mutagenized DNA, and the plasmid DNA which had the same structure as pLIOP3 was recovered as a mutant.

**Determination of nucleotide sequence.** The nucleotide sequence of the DNA fragment was determined by the dideoxy sequencing method (6) using phage M13mp10 as the cloning vector (7).

**Assay of β-galactosidase.** The β-galactosidase activity was measured by the method of SCHLEIF and WENSINK (8), using o-nitrophenyl galactopyranoside as a substrate.

**Purification of the repressor for the lactose operon.** The lac-repressor was purified following the method of ROSENBERG et al. (9) using the cells carrying plasmid pI which lacI was controlled by the thermolabile repressor (CIt857) of phage λ (see Fig. 1 for the structure of pI). The repressor was more than 95% pure according to analysis by SDS polyacrylamide gel electrophoresis.

**Binding assay of lac-repressor to lacPO.** Binding of the purified lac-repressor to lacPO was examined following the method of FRIED and CROTHERS (10) using agarose gel electrophoresis to detect the DNA fragment bound to the repressor.

**Isolation of lacI mutant.** *E. coli* WA802 or its polA derivative was transformed with a derivative of plasmid pSC101 (pJS213-5) which was constructed to carry the gene of chloramphenicol acetyltransferase (CAT; 11) under the control of lacPO. The plasmid was deleted with the par region (12) to make the plasmid unstable. Most of the chloramphenicol-resistant transformants were lacI mutants and the plasmid-free cell was easily recovered due to the instability of pJS213-5.

**General methods and chemicals.** Culture media, methods for bacterial transformation with plasmid DNA, agarose gel electrophoresis and DNA manipulations have been described previously (1, 13). The restriction endonucleases, T4 DNA ligase and T4 DNA polymerase were purchased from Nippon Gene or Takara Shuzo. Reactions with these enzymes were carried out as recommended by the supplier. Isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from Sigma Chemical Company. o-Nitrophenyl galactopyranoside was purchased from Nakarai Chemical Industry. Hydroxylamine hydrochloride was purchased from Wako Pure Chemical Industries, Ltd. The phage M13 sequencing kit N. 4502 and α-32P-dATP (800 Ci/mmol) were products of Amersham International plc.

**RESULTS**

**Construction and characterization of pLIOP3**

pLIOP3 which has lacI just behind lacPO is constructed following the scheme shown in Fig. 1. The lacI fragment was prepared from pMPC1 (3) so as to contain
the Shine and Dalgarno sequence but not its promoter and transcription terminator (14). BamHI adaptors were attached to the fragment as described in MATERIALS AND METHODS. This fragment was inserted at the BamHI site of pLOP3 to put ladl under the control of lacPO. The structure of pLIOP3 was confirmed by analysis with restriction endonucleases. Table 2 shows that pLIOP3
transforms wild-type *E. coli* but not the *polA* mutant. As shown previously, pLOP3 can not transform wild-type *E. coli* unless it carries F′lacI 

(MATERIALS AND METHODS). The expression was expressed as the number of transformants per µg of DNA.

Isolation and localization of mutations of pLOP3 that transform an *E. coli* polA strain

A mutant plasmid which could transform a *polA* strain was isolated by mutagenizing the plasmid as described in MATERIALS AND METHODS. Among several ampicillin-resistant colonies obtained after transformation, two colonies had plasmids whose structure was the same as pLOP3. One of them, named pLOP30, was examined further. The site of mutation was localized in the EcoRI-BamHI fragment (Fig. 1) carrying lacPO by replacing the fragment of pLOP3 with that of pLOP30 as shown in Table 2. This substituted plasmid was named pLMLOP3.

Nucleotide sequence of the lacPO region of pLMLOP3

The nucleotide sequences of the lacPO region of pLMLOP3 was determined and compared with those of the parent lacPO. The result shows that the mutant has two base substitutions at nucleotide number (no.) 10 (G→A) and no. 12 (G→A) (Fig. 2). These substitutions are known to make the operator constitutive (15). The other mutant had the same base substitutions, suggesting that these two mutants were derived from the same clone.
Character of the mutated lacPO

A structural gene of β-galactosidase isolated from pMC1403 was fused at the BglII site of gene O of pLmIOP3 (pLmIZ3). pLIZ3 and pBR-βZ were also constructed by inserting the structural gene of β-galactosidase at the BglII sites of pLIOP3 and pBR-βdv, respectively. Since the copy number of these plasmids is almost the same (45 copies), the activity of β-galactosidase synthesized by these plasmids will correspond to the transcription of the fused gene. With the mutant, the concentration of IPTG required for full induction was about 1/100 as much as for the parent (Fig. 3) and the activity was about fourfold higher. Even in the absence of IPTG, the mutant synthesized more β-galactosidase than was controlled by the pRoR promoter (pBR-βZ) but the synthesis by the parental plasmid was negligible.

The affinity of the mutated lacPO to bind with the purified lac-repressor was examined using agarose gel electrophoresis, as shown in Fig. 4. The affinity was about one fifth as much as the parental lacPO. This shows that the mutation made
the operator constitutive (O') (15).

Removal of the pBR322 origin from pLmIOP3

Since pLmIOP3 has the replication origin of pBR322, pLmI3 was prepared with the pBR322 origin removed. As shown in Table 2, pLmI3 transforms not only wild-type E. coli but also a polA mutant. The presence of F' lacI4 made the transformation efficiency about 10^6 as great as the control. pLmI3 was eliminated when the cells carrying both pLmI3 and pI, which had the lac-repressor under the control of the heat sensitive lacI repressor, were incubated at 42°C (Table 3). These results show that pLmI3 replicates using the replication machinery of λdv and is regulated by lac-repressor.

The host cells used in the above experiments have lac-repressor. A lacI mutant of E. coli WA802 polA was prepared and the effect of the host lac-repressor was examined. The result is shown in Table 4. pLmIOP3 gave transformants, but pLIOP3 did not. The mutation in lacPO seems essential for the pLIOP3 to multiply.
Stability and copy number of pLmI3

pLmI3 was not stably maintained and 50% was lost in 10 generations as shown in Fig. 5. The copy number of this plasmid was estimated from this figure to be about 2 assuming that it segregated randomly (16). It is also indicated that the copy number was less than 2 by the concentration of the plasmid DNA recovered from as a plasmid.

Table 3. Effect of lac-repressor on the maintenance of pLmI3.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Selective plate</th>
<th>Ratio of colony formers at 42°C at 30°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>pLmI3 and pI</td>
<td>Ap, Km</td>
<td>&lt;10⁻⁴</td>
</tr>
<tr>
<td>pLmI3₂</td>
<td>Km</td>
<td>8.6 x 10⁻¹</td>
</tr>
<tr>
<td>pI²</td>
<td>Ap</td>
<td>9.2 x 10⁻¹</td>
</tr>
<tr>
<td></td>
<td>Km</td>
<td>9.3 x 10⁻¹</td>
</tr>
</tbody>
</table>

_E. coli_ H1006 recA carrying pLmI3 and/or pI was plated onto Lb-agar containing appropriate drugs to select the cells carrying the intended plasmid, and was incubated at 30°C or 42°C. Colony formers at 42°C was compared with those at 30°C.

* Experimental controls.

Fig. 4. Affinity of lac-repressor to bind to the lacPO fragment. The digestion of pLIOP3 and pLmIOP3 by _PstI_ and _BamHI_ produces 5,390 bp, 1,368 bp and 931 bp fragments (see Fig. 1). The smallest fragment contains lacPO. The digest was incubated at 30°C for 20 min with the various concentration of lac-repressor indicated in the figure. They were electrophoresed in 1.5% agarose gel. The DNA bound with lac-repressor moved slower than the unbound molecule (10). Lanes 1 to 6: DNA fragments derived from pLIOP3. Lanes 7 to 12: those from pLmIOP3.

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* Experimental controls.

as a plasmid.
Table 4. Effect of \textit{lac}-repressor produced by the host on the transformation by pLIOP3.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>\textit{E. coli} WA802</th>
<th>\textit{polA}^+ \textit{lacI}^+</th>
<th>\textit{polA}^- \textit{lacI}^+</th>
<th>\textit{polA}^- \textit{lacI}^-</th>
</tr>
</thead>
<tbody>
<tr>
<td>pLIOP3</td>
<td>(6.2 \times 10^4)</td>
<td>(&lt;10)</td>
<td>(&lt;10)</td>
<td>(&lt;10)</td>
</tr>
<tr>
<td>pLmIO3</td>
<td>(5.1 \times 10^4)</td>
<td>(4.3 \times 10^4)</td>
<td>(3.2 \times 10^4)</td>
<td>(&lt;10)</td>
</tr>
<tr>
<td>pBR-\textit{idv}</td>
<td>(7.0 \times 10^4)</td>
<td>(2.0 \times 10^4)</td>
<td>(2.0 \times 10^4)</td>
<td>(&lt;10)</td>
</tr>
<tr>
<td>p344</td>
<td>(8.2 \times 10^4)</td>
<td>(&lt;10)</td>
<td>(&lt;10)</td>
<td>(&lt;10)</td>
</tr>
</tbody>
</table>

The effect of \textit{lac}-repressor produced by the host on the transformation frequency of pLIOP3 was examined using \textit{E. coli} WA802 \textit{polA lacI} which was prepared as described in MATERIALS AND METHODS.

(A)

(B)

Fig. 5. Segregation and the effect of IPTG on the growth of cells carrying pLmI3.

A: \textit{E. coli} WA802 carrying pLmI3 was grown in the absence of ampicillin in L-broth. The cells were withdrawn at appropriate intervals and the fraction of cells carrying pLmI3 was measured.

B: The killing of cells carrying pLmI3 by IPTG is shown here. One mM IPTG was added to \textit{E. coli} WA802 carrying pLmI3 at 0 hr and the growth of cells in the presence of ampicillin was measured. (-): The growth of cells carrying pLmI3 (no IPTG). (+): The surviving cells carrying pLmI3 after the addition of IPTG.

the cells carrying both pSC101 and pLmI3, when the copy number of pSC101 was assumed to be 5\cite{17}. These results suggest that although pLmI3 can multiply, its copy number is significantly smaller than \textit{idv} whose copy number is about 50\cite{18}.
Effect of the inducer

Addition of IPTG stimulated the replication of pLmI3 about 10-fold, and then the DNA had the usual twisted form (Fig. 6) and was biologically active. The transformation efficiency of the DNA extracted after induction was almost the same as that before induction (data not shown). The cells carrying pLmI3 were killed by the induction, probably due to the stimulated synthesis of P protein (1) (Fig. 5).

The minimum size required for replication

A minimum fragment of λdv which can multiply when O and P gene products are supplied in trans is composed of pRoR-tof-CII-ΔO where the O gene is cut at the EcoRI site (19). We prepared a derivative of pLmIOP3, pLmIori3, which consisted of lacP-lacI-ΔCII-ΔO where the O gene was cut at EcoRI. As shown in Table 5, this plasmid multiplied in E. coli WA802 polA when O and P gene products were supplied, but pLmIZ3 did not. The latter did not have the ori region of λdv (Fig. 3). This shows that pLmI3 uses O and P gene products and the replication origin of λdv for its replication.
DISCUSSION

The self-regulatory system of the pRoR-tof of ϕdv is replaced by artificially prepared lacPO-lacI which is supposed to be self regulatory. lac-Repressor is actually produced by this system under the control of lacPO, because plasmid pLIOP3 is maintained stably in wild-type (polA+) E. coli. pLOP3 can not become transformed unless the host carries F'lacI" (1). Though pLIOP3 transforms wild-type E. coli, it does not transform an E. coli polA mutant. A mutant which could transform the latter host was isolated by hydroxylamine mutagenesis.

The mutant had two base substitutions in lacPO which made for poor affinity of the operator with the repressor (Figs. 3, 4). Initiation of the transcription at the mutant promoter is higher than at pRoR. But transcription at parental lacPO is negligible in the absence of inducer (Fig. 3). The polA host was not transformed by pLIOP3 in the presence of IPTG sufficiently to induce the β-galactosidase of pLIZ3 to the level of pBR-ϕdv(I) by removing the EcoRI-HindIII fragment.

The results described here show that a self-regulatory system prepared artificially can function normally in vivo when appropriate modifications are introduced in it. Although the initiation of transcription at the mutated lacPO is higher than that of pRoR, the copy number of pLmI3 is smaller than ϕdv. There might be additional characters which made the pRoR promoter much more suitable for the DNA replication as a plasmid.

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