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Construction and characterization of a novel cross-regulation system for regulating cloned gene expression in *Escherichia coli*

(Recombinant DNA; repressor synthesis control; *tac* promoter; phage λ p_L promoter; dual repressor control)

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SUMMARY

A novel cross-regulation system employing two pairs of interacting promoter-repressor systems was constructed using the *tac-lacI* and λ p_L-cI promoter-operator-repressor systems. In particular, transcription of the *cat* gene and the fused *cI* gene is regulated by the *tac* promoter, while transcription of the *lacI* gene is controlled by the λ p_L promoter. In order to compare CAT production from this new system with a currently employed transcription control configuration, a control expression vector utilizing the constitutive repressor synthesis configuration was also constructed. In this construct, *cat* is under the control of the *tac* promoter, and *lac* repressor is provided from a single copy of the $lacI^q$ allele included in the plasmid. Induction results using different copy number vectors indicate that induced *cat* expression levels are at least twofold higher using the cross-regulation system which has very low basal expression. These results match well with previous mathematical modeling predictions indicating excellent control of basal expression and also higher cloned-gene expression post-induction over a broad range of copy numbers for a cross-regulation control configuration. Induction of the cross-regulation system both up-regulated the activation pathway and down-regulated the inhibition pathway, shifting the system steady-state from *lac* repressor expression to *cat* and *cI* expression. The control strategy presented here should be equally applicable to regulate transcription in diverse hosts.

INTRODUCTION

Recent advances in genetic techniques enable production of various cloned proteins in different organisms. Production of large quantities of these cloned proteins can be achieved by combining gene amplification and strong promoters. However, high expression of recombinant proteins has been shown to reduce host cell growth rate and, concomitantly, overall protein synthesis capability (Bentley et al., 1990). It is likely that competition between chromosomal-directed, host metabolic activity and plasmid-directed metabolic activity is a general contributor to this detrimental effect (Peretti and Bailey, 1987). Preferentially, production is delayed until the end of the growth phase when high expression of the cloned gene is achieved by induction or derepression (Park et al., 1991).

Cloned gene expression is governed by the rate of transcription, the stability of the mRNA, the rate of translation, and the stability of the protein. In particular, regulation of cloned gene transcription in *E. coli* has been

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Abbreviations: *A*, absorbance (1 cm); Ap, ampicillin; bp, base pair(s); βGal, β-galactosidase; CI, phage λ repressor; *cI*, gene encoding CI; CAT, chloramphenicol acetyltransferase; *cat*, gene encoding CAT; IPTG, isopropyl-β-D-thiogalactopyranoside; kb, kilobase(s) or 1000 bp; LB, Luria-Bertani (medium) (see legend to Fig. 4); PCR, polymerase chain reaction; PolIk, Klenow (large) fragment of *E. coli* DNA polymerase I; $p_L o_L$, major leftward early promoter and operator of phage λ (see Szybalski and Szybalski (1979); rpm, revolutions per min; S-D, Shine-Dalgarno (sequence); wt, wild type; XGal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; [ ], denotes plasmid-carrier state.

thoroughly studied for different promoter-operator systems including *lac* (Makoff and Oxer, 1991), *trp* (Latta et al, 1990), *tac* (Jaffe et al., 1988), and λ p_L (Mott et al., 1985). In each of these systems, transcription activity depends upon interactions with a specific repressor protein. For example, gene expression controlled by the λ p_L promoter is usually regulated by the temperature-sensitive CI repressor (encoded by ts-*cI*) (Remaut et al., 1983). Induction of cloned gene expression requires shifting the temperature from 28–30°C to 42°C (inactivates the ts-CI repressor). The main disadvantage of the temperature-induced system is the requirement for growth preinduction at a suboptimum growth temperature (28–30°C), resulting in reduced growth rate and increased contamination risks. Furthermore, temperature shift to 42°C activates the heat-shock response which results in significant changes in the protein composition of the cell, including elevated level of protease La which is implicated in the initial proteolytic attack on abnormally folded proteins (Goff and Goldberg, 1986; Kosinski and Bailey, 1991).

For expression systems utilizing the *lac, tac,* and *trp* promoters on multicopy vectors, a high basal level of expression is often observed in a wt strain in which only a single copy of the corresponding repressor gene is included in the chromosome (Stark, 1987; Latta et al., 1990). In this situation, multiple copies of these operators titrate out the wt level of repressor. This is particularly undesirable if the cloned product is toxic to the host. In order to alleviate this problem, a single copy of the repressor gene is often included in the expression vector, providing sufficient repressor to prevent expression before induction or derepression. Unfortunately, due to the high level of repressor, maximal induced expression levels are typically reduced in such constructs (Stark, 1987).

Recently, a new expression system design was proposed (Chen et al., 1991) based upon molecular-level mathematical modeling of the involved intracellular interactions. This novel cross-regulation system employed a dual repressor control configuration to regulate cloned-gene expression. In this arrangement, the repressor gene for the second promoter and the product gene are fused together with the first promoter to form an operon, while the expression of the first repressor gene is controlled by the second promoter. Product gene transcription is induced by addition of inducer which inactivates the first repressor. Simulation results using *lac* (first promoter) and p*R* (second promoter) promoter-repressor systems as examples indicated that this system retains good control of cloned-gene transcription before induction and also provides the highest transcription level post-induction (Chen et al., 1991).

The aim of present study was to construct and characterize a particular experimental realization of the cross-regulation system in *E. coli.* Because of their useful properties and the availability of the required genetic elements, the *tac-lacI* and p_L-*cI* promoter-repressor systems were chosen. The *cat* gene was used as a model reporter gene because of its stability in *E. coli* and existence of a sensitive assay for its activity. Applicability of the cross-regulation system over a broad range of plasmid copy numbers was explored by transferring the expression cassette into a series of closely related copy number plasmids.

RESULTS AND DISCUSSION

(a) Criteria for choosing the first and second promoter-repressor system

Although many different types of promoter-repressor systems can be used for the cross-regulation, there are at least two criteria that must be satisfied. These two criteria were determined from previous modeling efforts (W.C. and J.E.B., unpublished results), and they are presented here to serve as guidelines for future cross-regulation system design. There is no strict requirement for choosing the first promoter-repressor system as long as it is a fairly strong promoter. On the other hand, in choosing the second promoter-repressor system, it is important that the following criteria are satisfied: (*1*) the sensitivity of repression of this promoter by its repressor must be less than that of the first promoter; (*2*) the second promoter must be a stronger promoter than the first promoter. These two criteria are required to guarantee that the first promoter will be turned off in the pre-induction state. In general many systems can satisfy these two requirements. For this work, the *tac* promoter has been chosen as the first promoter because it is a conveniently available strong promoter and has been used to express many recombinant proteins in *E. coli* (De Boer et al., 1983; Amann et al., 1983). The p_L promoter is used as the second promoter because it is reported to be stronger than the *tac* promoter and has also been shown to satisfy the first criterion (Johnson et al., 1981).

(b) Construction of the constitutive repressor synthesis vectors (pCS series)

Plasmid pKC2 was constructed by inserting a 1.5-kb *Bsp*HI fragment from pTCAT (Khosla and Bailey, 1989) containing the *tac-cat* fusion into the corresponding complementary *Nco*I site in plasmid pSL1180 (Pharmacia). The resulting plasmid retained the entire *tac-cat* fusion including the strong *rrnB* termination sequence (this sequence is included in plasmid pKK223–3 (Pharmacia)) but did not extend into the *bla* gene region. Subcloning of the $lacI^q$ fragment into pKC2 was accomplished by

cleaving plasmid pMJR1560 (Amersham) with *Kpn*I + *Pst*I. A 1.2-kb fragment containing the entire *lacI*^{q} allele including its own promoter and transcription termination sequence was then subcloned into the corresponding sites in pKC2 to create pKC6 (Fig. 2). This construct contains the *lacI*^{q} and the *tac-cat* fusion facing in the opposite direction in order to minimize the possibility that any transcription initiated elsewhere on the plasmid could be extended into this region.

To transfer this expression cassette into different copy number plasmids, pKC6 was cleaved with *Bst*BI + *Sph*I, and a 3.1-kb fragment was ligated between the *Sac*I and *Sph*I sites of plasmids pDM246, pDM247, and pFH118 (Moser and Campbell, 1983) to give pCS246, pCS247, and pCS118, respectively. The *Bst*BI-*Sac*I sites were made blunt with PolIk and T4 DNA polymerase prior to ligation.

(c) Construction of the cross-regulation vectors (pCRR series)

The PCR technique (Saiki et al., 1988) was used to synthesize both the *lacI* and *cI* structural gene using plasmid pMJR1560 and plasmid pKB252 (Beckman and Ptashne, 1978) as templates, respectively. Primers I and II (Fig. 1a) were used to amplify the *lacI* gene. To synthesize the structural *cI* gene, primers III and IV were used (Fig. 1b). The 736-bp *cI* fragment obtained from PCR amplification was digested with *Eco*RI + *Pst*I, and ligated into pKQV4 (Strauch et al., 1989) (essentially the same as pKK223–3 except it also contained the *lacI*^{q} gene isolated from pMJR1560) previously opened with the same enzymes. The resulting plasmid pTCI carried the *cI* gene under the control of the *tac* promoter. Primer III used for the synthesis of this *cI* fragment was designed such that the distance between the start codon of the *cI* gene and the ribosome binding site (S-D sequence) on the plasmid is 10-bp long (recommended 10–15 bp for effective translation initiation according to Pharmacia; see Fig. 1c). Plasmid pSIAT was derived by replacing a 622-bp *Mlu*I-*Pst*I fragment carrying the entire *tac* promoter from pKC2 by a 922-bp *Bam*HI-*Pst*I fragment containing the *tac-cI* fusion with the *Mlu*I-*Bam*HI end rendered blunt by 'filling in' (using PolIk), thus creating a *tac-cI-cat* operon (Fig. 2). Both *Bam*HI and *Mlu*I sites were regenerated after ligation. The *lacI* PCR product was subjected to proteinase K treatment in order to improve the subcloning efficiency (Crowe et al., 1991). It was then subjected to phenol-chloroform extraction and ethanol precipitation. The purified 1169-bp *lacI* fragment was then ligated into the unique *Sma*I site of pUC18 to generate pUC18-lacI. To construct plasmid pλ-lacI, a 1.2-kb *lacI* fragment was obtained after cleaving pUC18-lacI with *Eco*RI + *Sph*I and inserted between the *Bsp*EI and

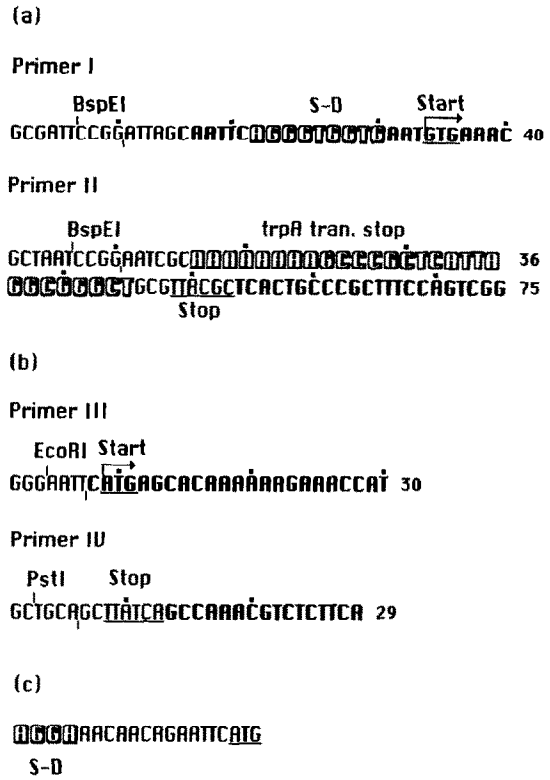

Fig. 1. Construction of cross-regulation system by PCR. (**a**) PCR primers I and II used for the synthesis of the *lacI* gene. The bold letters indicate the region of homology with the DNA template. The shadow letters indicate the S-D sequence of the *lacI* gene and the *trpA* transcriptional termination signal . The native *lacI* transcriptional termination signal which overlapped into the *lac* promoter region was replaced by a strong *trpA* transcriptional termination signal (Christie et al., 1981). In addition, an extra translational stop codon was inserted in frame after the original one. The S-D sequence for the *lacI* gene was also included in primer I. *Bsp*EI sites were created at both ends of the primers to facilitate subcloning of the amplified PCR fragment. All primers were synthesized at the Caltech Applied Microchemical Facility. (**b**) PCR primers III and IV used for the synthesis of the *cI* gene. The bold letters indicate the region of homology with the DNA template. The S-D sequence and the transcriptional termination signal were not included in these primers because they will be supplied from plasmid pKVQ4 used for subcloning the amplified *cI* fragment. Restriction sites *Eco*RI and *Pst*I were created at the 5′ end of primer III and IV, respectively. (**c**) Spacing between the start codon of the *cI* gene and the S-D sequence (shadowed) on plasmid pTCI is indicated.

*Sph*I sites of pPL-Lambda (Pharmacia) with blunt end ligation at the *Eco*RI and *Bsp*EI sites (both ends were made blunt by filling in with the PolIk). The resulting construct expressed the *lac* repressor under the control of the λ p_L promoter.

Plasmids pλ-lacI and pSIAT were cleaved with *Nru*I + *Bam*HI, and a 1.9-kb fragment from pλ-lacI was inserted into pSIAT, replacing the smaller fragment from the latter to yield pKC7. The resulting construct has the *tac-cI-cat* operon and the p_L-*lacI* fusion facing in the opposite orientation (Fig. 2). Since transcription termination signals were included at the 3′ end of the gene, it is unlikely that transcription initiated within the plasmid

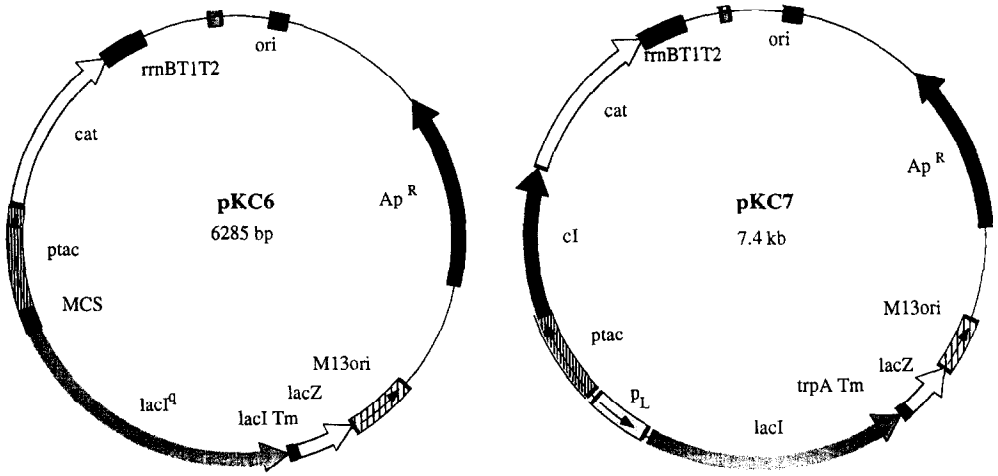

Fig. 2. Maps of the plasmids pKC6 and pKC7 carrying the constitutive repressor synthesis system and the cross-regulation system, respectively. Key: ptac: *tac* promoter; p_L: λp_L promoter; MCS: multi-cloning sites; Tm and T1T2: transcriptional terminators; ori: origin of DNA replication.

can be extended into this region. Consequently, it is likely that the expression of these genes would be under control of their own promoters. To transfer the expression cassette into the different copy number plasmids, pKC7 was digested with *Sac*II + *Nco*I, and a 3.4-kb fragment carrying the entire cross-regulation cassette was ligated into the same sites of the pCS series to obtain plasmids pCRR118, pCRR246, and pCRR247.

(d) The *lacI* and *cI* PCR fragments produce functional gene product

It is well known that DNA amplification using the PCR technique is susceptible to error due to the absence of the 3′ to 5′ proofreading exonuclease function in the *Taq* DNA polymerase (Kohler et al., 1991). Normally, DNA sequencing is the most reliable method to ensure that no error is incorporated into the amplified DNA fragment. However, in this work it is sufficient to verify that the gene fragments obtained from PCR produce functional gene products. A sensitive test for the presence of functional *lac* repressor is the ability to repress βGal synthesis in a *lacI*⁻ mutant that cannot provide its own *lac* repressor. Mutant CGSC808 (*lacI22*, *λ*⁻, *relA1*, *spoT1*, *thi-1*) (*E. coli* Genetic Stock Center) lacking the *lac* repressor forms blue colonies on an XGal plate due to the presence of βGal. On the other hand, this mutant forms white colonies on an XGal plate carrying pUC18-lacI, demonstrating that the *lac* repressor produced from the PCR *lacI* fragment is indeed functional (data not shown).

A similar experiment was carried out for the *cI* PCR fragment. In this experiment, plasmid pUC18cI, which carries the *cI* PCR fragment inserted between the *Eco*RI and *Pst*I sites of pUC18, was transformed into *E. coli* CY15050 (W3110*tnaAZ*/λt_L,*ΔlacU*169) (Kelly and Yanofsky, 1982). This strain carries a λ prophage and a temperature-sensitive *cI*857 gene. Shifting temperature from 30°C to 42°C destroys the temperature-sensitive repressor activity. The prophage DNA is detached from the host chromosome changing the prophage from lysogenic to lytic state, and eventually the host lyses. Fig. 3 shows that strain CY15050 carrying pUC18-cI can remain intact and continue growing after temperature shift, indicating that the PCR *cI* fragment produces active λ repressor to complement the host's temperature-sensitive λ repressor.

(e) The cross-regulation system functions as predicted based on computer simulation

For plasmid pKC7 (Fig. 2), the expression of the *cat* gene is under cross-regulation control. The XGal plating behavior of CGSC808[pKC7] (only white colonies were

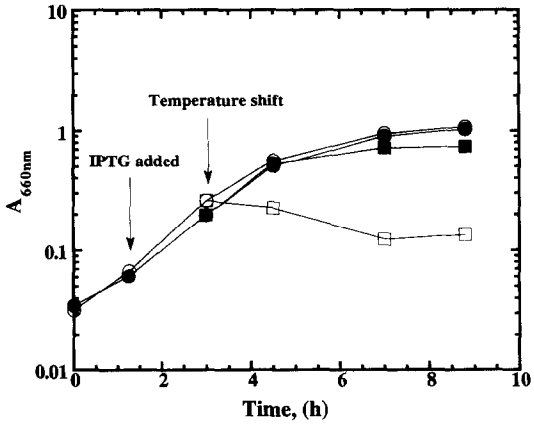

Fig. 3. Functional test for the PCR *cI* fragment. Strain CY15050 carrying plasmids pUC18 or pUC18cI were grown at 30°C. IPTG was added to 1 mM concentration at 1.5 h to induce expression of *cI* from plasmid pUC18-lacI. Half of each culture was then transferred to 42°C. Absorbance (A_{660}) was measured at 660nm with a Spectronic 21 spectrophotometer (Milton Roy). Symbols: (□), pUC18 at 37°C; (●), pUC18-lacI at 37°C; (□), pUC18 at 42°C; (■), pUC18-lacI at 42°C. The temperature-shift experiment was conducted in a New Brunswick GYROTORY water bath shaker model G76 at 250 rpm.

observed) indicates that the *lacI* gene under control of the p_L promoter supplies enough *lac* repressor for repression of multiple copies of the *lac* operator (both from the *tac* promoters on the plasmid and the *lac* promoter on the chromosome). This is in good agreement with simulation predictions indicating strong pre-induction repression of the first promoter, in this case the *tac* promoter (Chen et al., 1991). This is very important because, if this trend is reversed, the system will fail to control basal expression from the *tac* promoter, controlling instead the p_L promoter.

In order to quantify expression levels, we measured CAT production from the constitutive repressor synthesis configuration (pKC6) and also from the cross-regulation configuration (pKC7) in *E. coli* DH5α {F⁻, *endA*1, *hsdR*17($r_k^- m_k^+$), *supE*44, *thi*-1, λ^-, *recA*1, *gyrA*96, *relA*1, (*ϕ80dlac*am15)}. Before induction, both strains show a low level of CAT activity, with that of DH5α[pKC7] lower (Fig. 4). This finding is consistent also with model prediction of low preinduction transcription activity of the cross-regulation configuration. Upon induction by the addition of IPTG, the CAT activity increases significantly. However, the specific CAT activity for DH5α[pKC6] eventually levels off while that for DH5α[pKC7] continues to increase to approximately twice the level in DH5α[pKC6] after 3 h of induction. Since both systems utilize the same S-D sequence for the *cat* gene, the difference in expression is likely to be contributed from the difference in transcription.

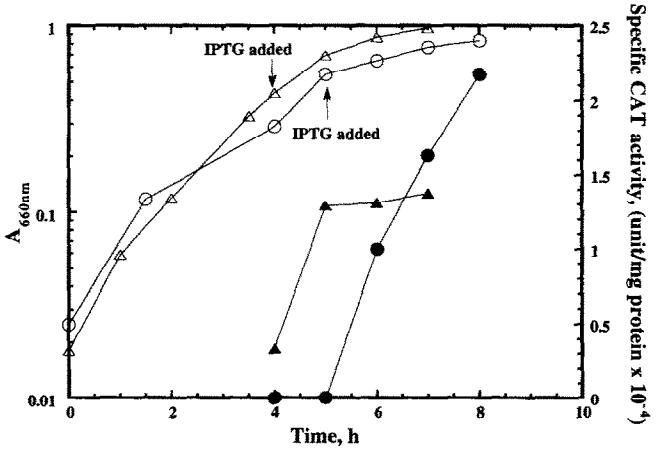

Fig. 4. Comparison of growth and CAT production for DH5α[pKC6] and DH5α[pKC7]. Cells were grown to approximately the same cell density ($A_{660} = 0.6$) before the addition of IPTG. Symbols: (○), A_{600} for pKC7; (△), A_{600} for pKC6; (●), CAT activity for pKC7; (▲), CAT activity for pKC6. LB medium containing 10 g Difco tryptone/5 g Difco yeast extract/10 g NaCl/3 g K_2HPO_4/1 g KH_2PO_4 (pH 7.0) (all per liter) was used for all growth experiments. Ap was added to a concentration of 50 μg/ml for selection. For induction of the *tac* promoter, IPTG was added to 1 mM concentration. CAT activity was determined with ^{14}C-labeled butyryl coenzyme A (New England Nuclear) according to recommended protocols (Newman et al., 1987). CAT activity is expressed in units of CAT per mg of total soluble protein.

A better indicator of the transcriptional activity is obtained by comparing the specific CAT synthesis rate of the two systems, which can be estimated from the slopes of a CAT vs. cell density plot. For stable enzymes, such a plot gives a straight line for any interval over which the specific enzyme synthesis rate and specific growth rate are constant. Under these conditions a mass balance on enzyme may be written:

$$\frac{dE}{dt} = kX = kX_o e^{\mu \Delta t} \tag{1}$$

where E is the enzyme activity per unit volume of culture, X is the cell density (A), k is the specific enzyme synthesis rate, μ is the specific growth rate, $\Delta t = t - t_o$ is the elapsed time from some starting time t_o, and subscript o denotes evaluation at t_o. Integrating this equation gives

$$E(t) = \frac{kX}{\mu} - \frac{kX_o}{\mu} + E_o \tag{2}$$

Data from the present experiments are plotted in this fashion in Fig. 5. Since the specific growth rates of both cultures are approximately constant and similar for A's in excess of 0.5, the higher slope for the DH5α[pKC7] indicates higher CAT specific expression for the cross-regulation vector. This is consistent with prior model simulations of the relative post-induction responses of the constitutive repressor and crossregulation transcription control designs.

Data from another experiment supporting the claim of higher *lac* repressor synthesis in the cross-regulation construct preinduction are shown in Table I. In this table, the βGal activity was measured for strains CGSC808 carrying plasmids pUC18, pMJR1560 and pλ-lacI as the source of *lac* repressor. As indicated, the strain carrying

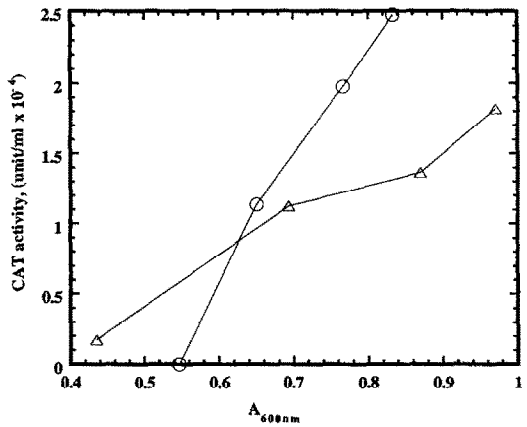

Fig. 5. Specific expression rate. CAT activities are plotted against corresponding cell densities for DH5α[KC6] (△) and DH5α[pKC7] (□). The specific expression rate is determined from the slopes of the plots.

TABLE I

βGal activity of strain CGSC808 carrying plasmids pUC18, pλ-lacI and pMJR1560

Strain	Source of *lac* repressor	βGal activity[a] (Miller units)
CGSC808[pUC18]	none	3000
CGSC808[pMJR1560]	$lacI^q$	60
CGSC808[pλ-lacI]	p_L-*lacI*	<0.1

[a] βGal was assayed at 30°C using *o*-nitrophenol-β-D-galactopyranoside as the substrate. Change in $A_{420}(\Delta A_{420})$ was monitored by a rate assay in a thermostated spectrophotometer (Shimadzu UV260). Specific activity is expressed as Miller units; 1 Miller unit = $100 \times \Delta A_{420}/\text{min} \times A_{600}$ (Miller, 1972). Cells were grown in shake flasks at 37°C and 275 rpm.

pUC18 shows a high level of βGal activity compared to the other two constructs. Examining these data, the βGal activity is lower for the pλ-lacI construct compared to that for pMJR1560. This is a strong indication that the amount of *lac* repressor provided from the p_L promoter is higher than that from the $lacI^q$ promoter, thus showing that more *lac* repressor was synthesized from the cross-regulation construct preinduction.

The dynamics of the cross-regulation system are illustrated in Fig. 6. Before induction, no band corresponding to the CAT protein is visible for either construct (Fig. 6, lanes 1 and 6). After IPTG addition, the CAT band immediately appears. For the cross-regulation system, bands for both *cI* repressor and CAT appear at the same time, showing that the synthetic operon constructed for the cross-regulation system is functional (Fig. 6, lane 7). However there is apparently more *lacI* protein from the control than the cross-regulation constructs (compare lanes 1 and 6, Fig. 6). This we believe is probably due to varying amount of proteins applied to the gel rather than a contradiction to our results shown in Table I.

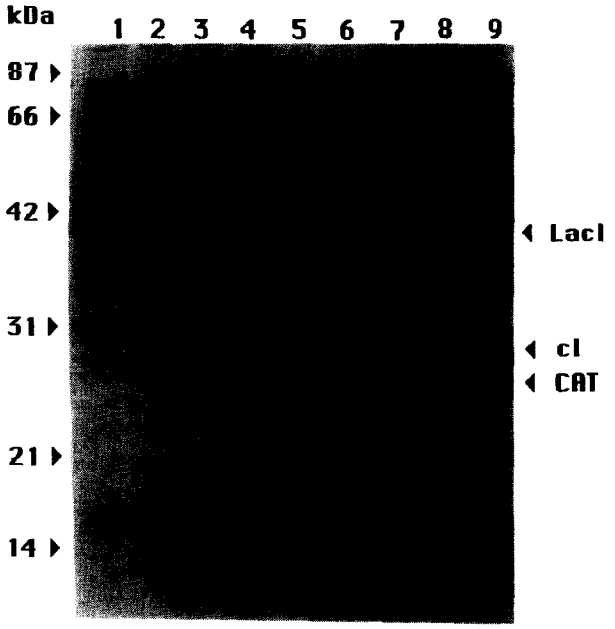

Fig. 6. Expression of CAT and repressor proteins from plasmids pKC6 and pKC7. SDS–PAGE was performed according to the method of Laemmli (1970). 0.1% SDS-12.5% PAGE analyses of total protein from cells carrying plasmid pKC6 (lanes 1–4) and plasmid pKC7 (lanes 6–9) are shown for the time immediately before (lanes 1 and 6) and 1, 2 and 3 h (lanes 2–4 and 7–9) after the addition of IPTG, respectively. Protein markers are shown in lane 5 and on left margin. Positions of the *lacI*, *cI* and *cat* gene products are indicated on right margin. Different amount of protein concentrations were used for these samples.

(f) Applicability of the cross-regulation system over a broad range of plasmid copy number

In order to demonstrate that this new configuration works equally well over a broad range of plasmid copy number, the expression cassettes for both the cross-regulation system and the constitutive repressor synthesis system were transferred to a series of very similar but different copy number vectors. These vectors only differ in their source of RNAI which is an inhibitor of replication for the ColE1-type replicon (Moser and Campbell, 1983). Plasmid content measurements (Table II) obtained from densitometry scan of photographic negatives are in qualitative agreement with those observed previously (Seo and Bailey, 1985). As indicated in previous experiments with these different copy number vectors, the specific growth rate of *E. coli* carrying these vectors decreases significantly at high copy number (Seo and Bailey, 1985). The same trends were also observed here for both configurations as can be seen in Table II. Approximately the same decrease in growth rate is observed for both configurations before induction, indicating basal expression of the CAT protein does not play an important role in the reduction of growth. On the other hand, growth is much more severely reduced for the cross-regulation configuration after the addition of IPTG, reflecting the redirection of the cell resources towards CAT production. This reduction increases with increasing copy number (data not shown). Induction experiments reveal that the cross-regulation system consistently produces at least twice the specific CAT level compared to the constitutive regulation system independent of copy number (Table II). More importantly, the basal levels of CAT expression from the cross-regulation system are consistently lower than those obtained from the control system (in the range of 0.01% of total protein). Results from all these induction experiments exhibit very similar trends with time post-induction as those observed with the pKC6 and pKC7 constructs. For the cross-regulation system, the CAT level continues to increase up to 7 h post-induction (data not shown).

TABLE II

Comparison of specific growth rates and CAT expression for strain HV101 carrying the pCRR and pCS vectors grown in LB medium

Vector[a]	Copy number[b]	Specific growth rate[c] (h^{-1})		Induced CAT level[d] (g/g protein)	
		pCRR	pCS	pCRR	pCS
pCS247	50	0.772	0.774	0.0192	0.0085
pCS246	90	0.770	0.781	0.0339	0.0096
pCS118	150	0.704	0.711	0.0435	0.0190

[a]For descriptions of these vectors, see section **b**.

[b]Plasmid copy number was determined essentially by the method of Projan et al. (1983). The plasmid copy number was calculated by multiplying the peak height ratio by the bp number ratio of chromosome to plasmid.

[c]Shake flask experiments were carried out at 250 rpm in a New Brunswick INNOVA 4000 shaker at 37°C. Cells were grown until $A_{600} = 1$ and the culture was then split into two halves. To one, IPTG was added to 1 mM concentration for induction of CAT production.

[d]Cells were disrupted by sonication. The soluble fraction was used for protein and CAT analysis. Total protein concentration was determined using a Sigma kit (No. P5656). Total CAT content was measured by a CAT ELISA kit obtained from 5 Prime, 3 Prime, Inc.

(g) Conclusions

(*1*) The novel cross-regulation system constructed in this work not only retains excellent control of basal expression, but it also achieves a higher induced level of cloned protein production. Induction enables this system to both up-regulate its activation pathway and down-regulate its inhibition pathway. Although no such system has been reported in bacterial cells, a very similar type of dual cross-regulation control was recently reported for the hormone-sensitive adenylyl cyclase system in rat cells (Hadcock et al., 1990; 1991). Our results can be summarized in a model presented schematic in Fig. 7. The addition of IPTG is believed to have two major effects. First, the formation of a IPTG-*lac* repressor complex results in an increase in the transcription activity from the *tac* promoter. This is demonstrated by an increase in CAT and CI repressor levels. Increase of the intracellular CI repressor concentration then cross-regulates the p_L promoter and turns off transcription initiating there. The net result is a lower *lac* repressor concentration as demonstrated in Fig. 6.

(*2*) Results presented in this study demonstrate a new kind of expression control concept. It is reasonable to expect that this type of configuration can be applied using a wide variety of different promoter systems and hosts and is not restricted to the example illustrated in this study. The success of these experiments also validates the utilization of molecular level mathematical models for discovering novel genetic designs with practical utility. Work is currently in progress in our laboratory to determine the factors affecting recombinant protein yield using the cross-regulation system.

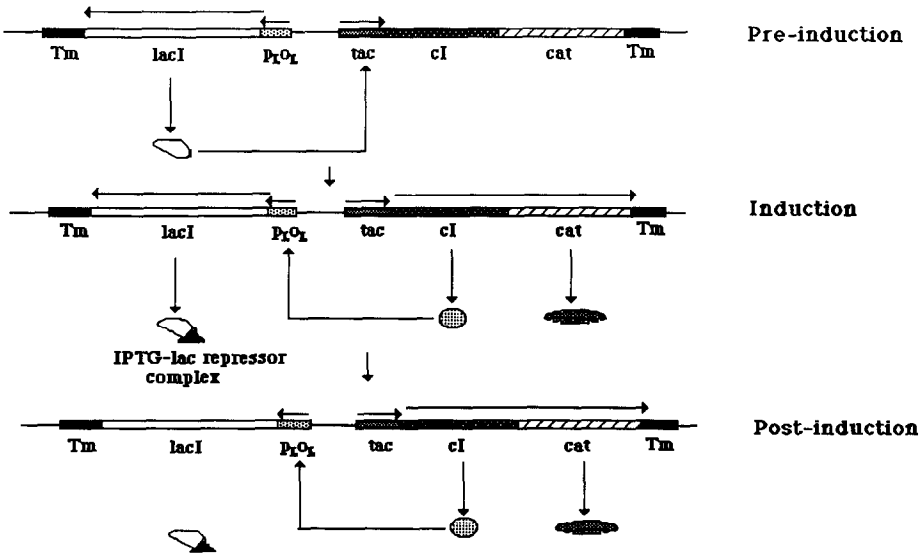

Fig. 7. A model representing the effect of IPTG addition to the cross-regulation system. Before induction, sufficient amount of *lac* repressors are available to turn off transcription from the *tac* promoter. After induction by the addition of IPTG, transcription is initiated from the *tac* promoter. One of its gene products, *cI*-encoded repressor, then cross-regulates and turns off transcription from the p_L promoter. For description of symbols, see Fig. 2.

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