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Independent and tight regulation of transcriptional units in *Escherichia coli* via the LacR/O, the TetR/O and AraC/I_1-I_2 regulatory elements

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ABSTRACT

Based on parameters governing promoter activity and using regulatory elements of the *lac*, *ara* and *tet* operon transcription control sequences were composed which permit the regulation in *Escherichia coli* of several gene activities independently and quantitatively. The novel promoter $P_{LtetO-1}$ allows the regulation of gene expression over an up to 5000-fold range with anhydrotetracycline (aTc) whereas with IPTG and arabinose the activity of $P_{lac/ara-1}$ may be controlled 1800-fold. *Escherichia coli* host strains which produce defined amounts of the regulatory proteins, *Lac* and *Tet* repressor as well as AraC from chromosomally located expression units provide highly reproducible *in vivo* conditions. Controlling the expression of the genes encoding luciferase, the low abundance *E.coli* protein DnaJ and restriction endonuclease *Cfr*9I not only demonstrates that high levels of expression can be achieved but also suggests that under conditions of optimal repression only around one mRNA every 3rd generation is produced. This potential of quantitative control will open up new approaches in the study of gene function *in vivo*, in particular with low abundance regulatory gene products. The system will also provide new opportunities for the controlled expression of heterologous genes.

INTRODUCTION

Genetic switches which permit the control of individual gene activities quantitatively and specifically will greatly facilitate the study of gene function *in vivo*. They would be particularly useful for the analysis of phenotypes which arise through small perturbations of sensitive equilibria. The signalling pathway of the heat shock response (1) or the control of cell division (2) may be just two of many examples.

In the past, regulated promoters of the *Escherichia coli* system such as P_L of phage lambda and the promoter of the *lac* operon as well as some of its derivatives have been widely used to control gene expression (3–5). Moreover, the specialized RNA polymerase/promoter system of phages T7 and T3 was applied when particularly tight control appeared to be required (6,7). While useful in a great number of applications, these systems have serious limitations. Thus, P_L is commonly induced by inactivating the repressor cI 857 via a temperature shift. This induction principle does not permit quantitative control over time and, in addition, causes pleiotropic effects. Similar limitations exist for experimental schemes where the introduction of, for example, phage T7 RNA polymerase into a cell via phage infection activates a gene (8). On the other hand, the promoter of the *lac* operon, P_{lac}, a well regulatable promoter of intermediate strength depends on the activation by CRP/cAMP. This activating complex affects, however, many additional operons and thus profoundly changes the metabolic state of the cell when switched into its active form by cAMP. The P_{lac} derivative, P_{tac} (4) and similar constructs like P_{trc} or P_{tic} (9) which do not depend on activation are repressed to a reasonable extent only at *Lac* repressor concentrations which hardly allow full induction. The more recently described systems where promoters of the *ara* (10) and the (Tn*10*) *tet* operon (11) were employed are useful alternatives; their range of regulation and their tightness in the repressed state may, however, fall short when compared with the system described here although we have not performed direct comparisons.

Here we describe a system for the quantitative and independent control of two transcription units in *E.coli*. The centerpiece of the system are regulatable promoters which were developed following principles described earlier (12). They are controlled by elements of the *lac*, *ara* or *tet* (Tn*10*) operon and, accordingly, promoter activities are sensitive towards IPTG, arabinose or tetracycline, respectively. These promoters are tightly repressible and can be regulated over an up to 5000-fold range. By varying the plasmid copy number the regulatory range of these promoters can be shifted to span different windows. *Escherichia coli* strains which produce defined amounts of *Lac* and *Tet* repressor (*Lac*R, *Tet*R) as well as of AraC ensure reliable intracellular conditions. The tightness of the system is demonstrated by quantitative control of a low abundance protein of *E.coli* as well as by the stable maintenance of a gene encoding a restriction endonuclease. This endonuclease is upon induction efficiently overproduced despite of the immediate growth arrest of the culture.

MATERIALS AND METHODS

Construction of the pZ vector system

Modules II and III of the pZ vectors (Fig. 2) were synthesized by PCR (13) using various templates. The resistance genes were amplified together with their genuine promoters by primers

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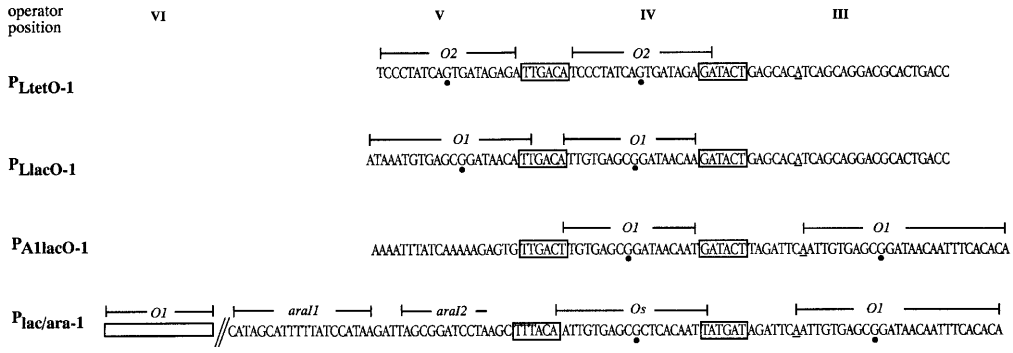


Figure 1. Topography and sequences of promoters. All promoter sequences which are aligned via the –10 hexamer are flanked by *Xho*I (upstream) and *Eco*RI (downstream) cleavage sites whose sequences are not shown. The –10 and –33 hexamers are boxed and the transcriptional start site is underlined. Bars indicate the *lac* (*lac*O) and the *tet* (*tet*O) operators as well as the I_1-I_2 binding site of AraC. O1 denotes the corresponding operator sequence in the *lac* operon, Os is a symmetrical 20 bp synthetic operator (15). O2 indicates the corresponding operator sequence in the Tn*10* tetracycline resistance operon (50). An additional O1 sequence is indicated further upstream (at –448) of $P_{lac/ara-1}$. The central base pair of the operators is indicated. The roman numbers III–VI give the positions of the operators relative to the promoter.

which introduced the flanking restriction sites *Sac*I and *Aat*II. Similarly, ColE1 and p15A origins of replication were produced to be flanked by restriction sites *Avr*II and *Spe*I except for the replication region of pSC101 or pSC101* which contain an internal *Spe*I site (Fig. 2). The following plasmids served as templates to retrieve various components for the pZ plasmid family: pDS12 (14) for the ColE1 origin, the terminators T1 of the *rrn*B operon and t_0 of phage lambda as well as the gene conferring resistance to ampicillin (Ap); pDM1.1 (15) for the p15A origin and the kanamycin (Kn) resistance gene; pACYC184 (16) for the gene encoding chloramphenicol (Cm) resistance; pBluc (17) for the luciferase gene; pSC101 (18) for the pSC101 origin; pBB1 (B. Bukau, unpublished) for the gene conferring resistance to Spectinomycin (Sp); pLDR11 (19) for the lambda *attP* site. The pSC101* origin is derived from the origin of replication of pMPP6 (20) by PCR using a proper mismatch primer.

To assemble the integration vector pZS4Int-1 the *laci*^{q} allele was retrieved from pDM1.1 (15), the *tetR* gene from pDM1.700 (21) and promoter P_{N25} from pDS1 (22). The sequences of representative members of the pZ vector family were submitted to the GenBank database (accession numbers U66308–U66313).

Construction of promoters

Promoters $P_{LlacO-1}$, $P_{A1lacO-1}$, $P_{LtetO-1}$ and $P_{lac/ara-1}$ were obtained by total synthesis. The *lac* operator *O1* upstream of $P_{lac/ara-1}$ was introduced via a PCR primer with the corresponding overhang and cloned as a 5′-*Aat*II–*Xho*I-3′ fragment upstream of the promoter. The intervening sequence between the promoter and the upstream operator was derived from the human *c-myc* gene (23) to minimize recombination and potential transcriptional signalling. After cloning, all promoter sequences were verified by dideoxy sequencing (24).

Cloning of the restriction endonuclease *Cfr*9I

The gene encoding restriction endonuclease *Cfr*9I was amplified from vector pCfr9I2.3X (25) by PCR and cloned into vectors pZS*24 and pZA24, respectively, via *Kpn*I/*Xba*I or *Eco*RI/*Xba*I. The utilization of the *Kpn*I cleavage site resulted in a mRNA with a strong RBS, whereas the RBS generated via *Eco*RI was ~10 times less efficient.

Construction of *E.coli* strain DH5αZ1

Escherichia coli strain DH5αZ1 was obtained following the description of Diederich *et al.* (19). For integration of plasmids of the pZ series into the chromosome, the lambda *attP* site pLDR8 was cloned into the *Avr*II site of pZS4Int. For integration, the origin of replication was removed by cleavage with *Spe*I and *Avr*II (generating compatible cohesive ends) and the religated fragment was transferred to *E.coli* DH5αpLDR8 by electroporation. Cells were incubated for 2 h at 42°C and then at 37°C overnight and transformants were selected on LB Sp[50 µg/ml] plates.

Determination of *in vivo* promoter activities

Promoters $P_{LlacO-1}$, $P_{LtetO-1}$ and $P_{lac–ara-1}$, respectively, were inserted into plasmids of the pZ series and the expression of the eukaryotic *luciferase* gene of *Photinus pyralis* (17,26) was measured by monitoring its enzymatic activity. Overnight cultures of *E.coli* cells DH5αZ1 grown at 37°C in LB medium containing the appropriate antibiotics were diluted 1:100 in LB medium in presence or absence of various inducers [1 mM IPTG, L(+)-arabinose, anhydrotetracycline] at concentrations indicated. After 3 h, the OD_{600} was measured and the cultures were kept at room temperature for 15 min. To determine luciferase activities in crude extracts of logarithmically growing cultures 3 ml cells were sedimented, resuspended in 50 µl lysis buffer (1 mM EDTA, 1 mg/ml lysozyme) and incubated at room temperature for 15 min. Upon addition of 300 µl H_2O and 300 µl buffer I (100 mM KH_2PO_4, 1 mM DTT, pH 7.8) 35 µl were mixed with 250 µl buffer II (15 mM $MgSO_4$, 25 mM glycylglycin, 2.5 mM ATP) and luciferase activity was measured (10 s, delay 0 s) in a Berthold Lumat type LB9501. Activities are given as ‘relative light units’ (RLU) after subtraction of the instrumental background and normalization to the number of viable cells (27).

Enzymes, antibodies, media and chemicals

Standard DNA manipulations were carried out as described (30). All enzymes were purchased from Boehringer Mannheim. DNA sequencing reactions were performed using the Pharmacia T7 sequencing kit. Synthetic oligonucleotides and sequencing primers were supplied by the inhouse facility. Antibiotics were added to the growth medium at the following concentrations: 100 µg/ml

ampicillin, 40 µg/ml kanamycin; 25 µg/ml chloramphenicol and 50 µg/ml spectinomycin. Luciferin, IPTG and standard chemicals, p.a. grade, were purchased from AppliChem, L(+)-arabinose from Sigma while anhydrotetracycline was obtained from Acros. Radiochemicals were purchased from Amersham & Buchler.

Anti-DnaJ rabbit serum for immunoblots, prepared in house, was diluted 1:7500 for the preparation of immunoblots. Specific antibody–DnaJ complexes were detected with alkaline phosphatase-conjugated anti-rabbit IgG (Promega) as described (29).

RESULTS

Rational of promoter designs

The decisive parameter for the efficient repression of promoters where repressors interfere directly with the binding of RNA polymerase is the rate of complex formation (k_{ON}) between RNA polymerase and promoter (15). Promoters which bind RNA polymerase at low rates are well repressed since they give the binding of the repressor a competitive advantage. Such promoters, however, remain weak upon induction unless they are activated as, for example, is the case for P_{lac}. By contrast, promoters which are strong in the absence of any activator bind RNA polymerase efficiently and can in general not be well repressed. We have developed two classes of repressible promoters: those which, after combination with operators, still initiate RNA synthesis efficiently and those which require activation in the derepressed state. The first class is derived from strong phage promoters such as P_L of phage lambda (31) and P_{A1} of phage T7 (32). Members of the second class are derivatives of P_{lac}. Sequences of the *lac* or *tet* operator were inserted within the various promoters at positions previously shown to be most effective (15), particularly in the downstream or within the spacer region, position III and IV, respectively (Fig. 1). Moreover, in some constructs the effect of auxiliary operators of the *lac* system was exploited by placing a third *lac* operator sequence in position VI upstream of the promoter (Fig. 1). For activating 'low k_{ON} promoters', AraC has been utilized which in contrast to CRP/cAMP acts highly specifically.

Construction of promoters controlled by *Tet*R or *Lac*R

Promoter P_L of phage lambda has a low homology score and binds RNA polymerase with a moderate forward rate constant of 1.1×10^7 M^{-1} s^{-1} (31). It is a strong promoter *in vivo* which, nevertheless, can be tightly repressed by cI, the lambda repressor. We have replaced the cI binding sites with sequences encoding the operator 2 (*tet*O2) of the Tn*10* tetracycline resistance operon (33). The resulting 74 bp promoter–operator sequence, $P_{LtetO-1}$, obtained by oligonucleotide synthesis contains a *tet*O2 sequence in position V and a 18 bp *tet*O2 core sequence in the spacer region (Fig. 1). $P_{LtetO-1}$ is tightly repressible by the *Tet* repressor and can be regulated over an up to 5000-fold range by supplying anhydrotetracycline (aTc) to the culture (Table 1a). In an analogous way, *lac*O1 sequences were integrated into P_L (Fig. 1): an 18 bp sequence in the spacer region (overlapping by 1 bp with the –33 hexamer) and a 22 bp sequence upstream of the promoter centred around position –43 (overlapping by 2 bp with the –33 hexamer). The activity of the resulting promoter $P_{LlacO-1}$ can be regulated over a >600-fold range by IPTG in *E.coli* DH5αZ1 (Table 1a).

Previously we have modified P_{A1} of phage T7 in a similar fashion (Lanzer and Bujard, unpublished) by inserting two *lac* operator sequences into position III and IV, i.e. into the spacer and the downstream region (Fig. 1). This strong promoter binds RNA polymerase with a relatively high forward rate constant (34) and although the *lac* operator sequence in position III reduces the rate of promoter clearance (22), $P_{A1lacO-1}$ remains a strong promoter *in vivo* when derepressed. At *Lac* repressor concentrations as in *E.coli* DH5αZ1, this promoter can be regulated over a 350-fold range by IPTG (Table 1a).

Table 1. (**a**) Induction and repression of $P_{LtetO-1}$, $P_{LlacO-1}$ and $P_{A1lacO-1}$ in *E.coli* DH5αZ1. (**b**) Induction and repression of $P_{lac/ara-1}$ in *E.coli* DH5αZ1

a

Promoter	replication origin of Vector	copy number in log Phase	Promoter strength (RLU/cell x 10^{-4}) - aTc	Promoter strength (RLU/cell x 10^{-4}) + aTc	Regulatory range
$P_{LtetO-1}$	ColE1	50 - 70	11	27900	2535
	p15A	20 - 30	3,5	12850	3670
	pSC101*	3 - 4	0,4	2020	5050
			- IPTG	+ IPTG	
$P_{LlacO-1}$	ColE1	50 - 70	35	21630	620
$P_{A1lacO-1}$	ColE1	50 - 70	30	10430	350

b

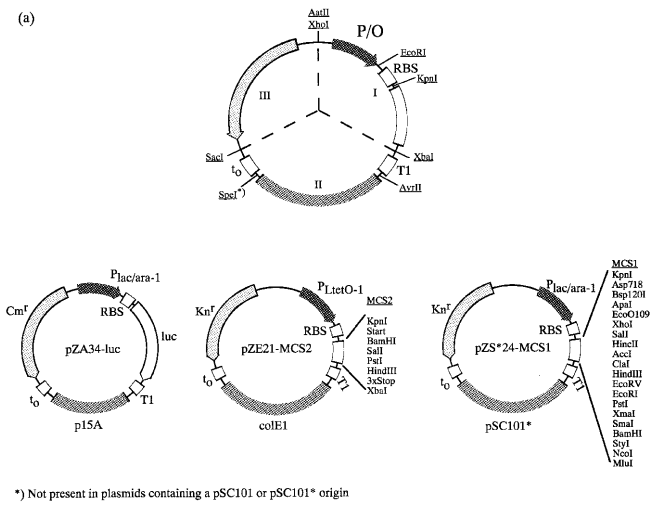
Promoter	replication origin of Vector	copy number in log phase	Promoter strength (RLU/cell x 10^{-4}) - IPTG - Arab.	+ IPTG - Arab.	+ IPTG + Arab.	Regulatory range
$P_{lac/ara-1}$	ColE1	50 - 70	7	875	12400	1770
	p15A	20 - 30	2,8	230	4950	1765
	pSC101*	3 - 4	0,6	41	1130	1885

Promoters were inserted upstream of the luciferase gene in the pZ vectors containing the origin of replication indicated. The various constructs were transfered into *E.coli* DH5αZ1. Overnight cultures of such transformants were diluted 1:100 in LB medium and grown up in presence or absence of aTc or IPTG, respectively. The concentration of aTc was 100 ng/ml, of IPTG 1 mM. At OD_{600} = 0.5, cells were harvested and luciferase activity was determined. The luciferase activities given are the mean values of five independent experiments (standard deviation <10%). The intracellular copy numbers were determined by comparing luciferase activity of cells harbouring the respective plasmids with the activity in cells containing only a single luciferase expression unit integrated in the chromosome (data not shown). They agree well with previously published data derived from direct copy number measurements (14). In Table 1b, $P_{lac/ara-1}$ was induced either by 1 mM IPTG alone or by 1 mM IPTG and L(+)-arabinose (0.05%).

A promoter controlled by *Lac*R and AraC

P_{lac}, when derepressed by IPTG and activated by CRP/cAMP, is a promoter of intermediate activity *in vivo* (32). Some mutants of P_{lac} show increased activity *in vivo* but remain susceptible to repression as well as activation. One of these mutant promoters is P_{lac-8A}. It differs from the wild type by a single T to A base change at position –8 (Fig. 1) and has a 3-fold higher promoter strength *in vivo* when compared with P_{lac} (35). Since P_{lac-8A} has still a low homology score and consequently binds RNA polymerase rather slowly, it is tightly repressible and can be activated by

(a)



) Not present in plasmids containing a pSC101 or pSC101 origin

(b)

origin of replication		resistance marker		regulatory unit		designation of vector
E	ColE1	1	Ampicillin	1	$P_{LtetO-1}$	pZE11-
A	p15A	2	Kanamycin	2	$P_{LlacO-1}$	pZA22-
S	pSC101	3	Chloramphenicol	3	$P_{A1lacO-1}$	pZS33-
S*	pSC101*	4	Spectinomycin	4	$P_{lac/ara-1}$	pZS*44-

Figure 2. The pZ vector system. (**a**) Overall outlay. The plasmids are composed of three modules which are separated by the unique cleavage sites *Xho*I/*Aat*II, *Xba*I and *Sac*I as indicated in the scheme in the upper part. Module I contains the signals for transcriptional regulation, i.e. promoter/operator constructs as well as a ribosomal binding site (RBS) which both can be exchanged by unique cleavage sites [*Xho*I(*Aat*II)/*Eco*RI or *Eco*RI/*Kpn*I, respectively]. This module contains also one of two multiple cloning sites (MCS 1, 2) for the integration of a gene of interest. Module II harbours one of four origins of replication (ColE1, p15A, pSC101, pSC101*). They are shielded from readthrough transcription by terminator T_1 of the *rrnB* operon and t_0 of phage lambda. Module III contains one of four antibiotic resistance markers which carry their genuine promoters and ribosomal binding sites. The lower part shows some standard pZ plasmids with their designation as explained in (b). (**b**) Nomenclature of the pZ vector system. The second letter of the pZ plasmid denotes the origin of replication (E through S*) and the first number indicates the resistance marker (1–4). The second number (1–4) defines the promoter controlling the transcription of the gene of interest. The MCS or the description of the gene of interest follows this code as exemplified for the three plasmids in (a).

CRP/cAMP (35). To convert P_{lac-8A} into a well regulatable promoter, we have introduced three modifications. First, a symmetrical 20 bp *lac* operator sequence (Os, Fig. 1) was placed in the spacer region. Second, a 35 bp wild-type operator sequence (*lac*O1) was integrated upstream of the promoter at position –448 following principles described previously (36–38). Third, the CRP/cAMP binding site was deleted and replaced by the I1/I2 recognition site of AraC, the repressor–inducer of the BAD promoter of the *ara* operon (39). To maintain the –35 hexamer of P_{lac-8A} and to centre the I_1/I_2 sequence around –53 as in the *ara* operon, 5 bp of the I_2 site were abolished. The resulting $P_{lac/ara-1}$ was examined for its regulatory potential in *E.coli* as described below. As shown in Table 1b, this promoter can be regulated over an ~1800-fold range whereby derepression via IPTG causes an ~100-fold and activation via arabinose a 15–20-fold increase in promoter activity.

The pZ vector system

The vectors depicted in Figure 2 emerged from our earlier developments, the pBU (40), pDS (14) and pUH (Lanzer and Bujard, unpublished) series. The salient feature of the pZ plasmids is their modular structure. Module I contains all the regulatory elements which control the expression of a gene of interest i.e. the regulatable promoter, a ribosomal binding site (RBS) and a transcriptional terminator. In the basic pZ plasmid, this module harbours multiple cloning sites (MCS). The transcriptional signals as well as the RBS can be exchanged using unique cleavage sites. Module II contains an origin of replication which is protected from outside transcriptional readthrough (14) by two terminators. Four origins of replication were adjusted to fit into the system via unique cleavage sites. This permits the variation of the plasmid copy number as well as of the compatibility group. Thus, when the ColE1, the p15A or the pSC101 origin of replication is used, intracellular copy numbers of 50–70, 20–30 and 10–12, respectively, are established. Particularly low copy numbers are achieved with the origin of pMPP6 (20), a derivative of pSC101 which gives rise to only three to four plasmids per cell and is referred to in our system as pSC101* origin. Finally, module III carries a resistance marker and the genes encoding ampicillin, kanamycin, chloramphenicol and spectinomycin resistance together with their genuine transcription and translation signals were again adjusted to fit into the constructs via unique cleavage sites. The nomenclature of the pZ plasmid family is explained in Figure 2. Of particular interest for the study here were the plasmids which contain a modified luciferase gene (17) as reporter for promoter activity. The effect of using different origins of replication led to a 15–20-fold shift of the regulatory window. This is most clearly demonstrated by comparing the luciferase activities in the repressed state of $P_{LtetO-1}$ and $P_{lac/ara-1}$ when integrated into pZE, pZA and pZS* (Table 1).

The *E.coli* host strain DH5αZ1

To ensure stable and defined conditions for the synthesis and maintenance of the regulatory proteins *Tet*R and *Lac*R, the genes encoding these two repressor molecules were placed under the control of the two constitutive promoters P_{N25} and the $lacI^q$ promoter P_iq (41), respectively, and integrated in tandem into the chromosome of *E.coli* strain DH5α at the phage lambda attachment site (42) as outlined in Figure 3. Analysis of several spectinomycin-resistant colonies by Southern blot analysis (data not shown) showed that the two transcription units encoding *Tet*R and *Lac*R as well as the spectinomycin resistance marker were stably integrated in the DH5α genome. The resulting strain, DH5αZ1, produces ~3000 molecules of *Lac*R and around 7000 molecules of *Tet*R per cell as determined by ELISA and Western blot (data not shown). Since *E.coli* DH5αZ1 is a genuine producer of AraC, all regulatory proteins required are constitutively synthesized in the cells which were used throughout the experiments described here. The entire unit encoding *Lac*R, *Tet*R and Sp^r can be readily transferred to other *E.coli* strains by phage *P1* transduction as exemplified for the widely used W3110 strain which led to *E.coli* W3110Z1 (data not shown).

Regulation of the activity of promoters $P_{LtetO-1}$, $P_{LlacO-1}$, $P_{A1lacO-1}$ and $P_{lac/ara-1}$

Promoter $P_{LtetO-1}$ is controlled by the operator repressor system of the Tn*10*-derived *tet* resistance operon. Accordingly it is

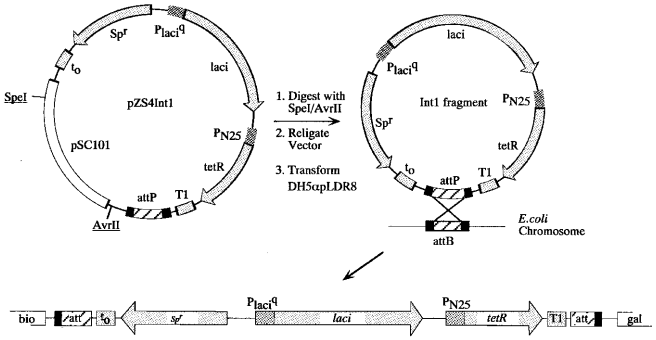


Figure 3. Integration of transcription units encoding *Lac*R and *Tet*R into the *E.coli* genome. The two repressor-encoding genes arranged in tandem and controlled by the promoter P_{laci^q} and P_{N25} respectively were inserted into pZS4Int1 carrying the phage lambda attachment site at *attP*. Upon cleavage by *SpeI*/*AvrII* and removal of the origin of replication, the compatible ends were ligated. *Escherichia coli* cells containing plasmid pLDR8, a thermosensitive plasmid encoding lambda integrase (19), were transformed with the ligated DNA and after incubation at non-permissive temperatures spectinomycin-resistant clones were selected and examined for the presence of the *lac*i and *tet*R transcription unit. The arrangement of these units in the chromosome is shown in the lower part. Terminators t_0 and T_1 prevent transcription from the integrated promoters into the neighbouring regions of the *E.coli* genome. The transcription units can be readily transferred to other *E.coli* strains by phage *P1* transduction.

induced by tetracyclines of which anhydrotetracycline is presently the most suitable one. The other promoters are all induced by IPTG and $P_{lac/ara-1}$ can, in addition, be activated by L(+)arabinose.

The regulatory range of all promoters was determined using the luciferase gene as reporter unit in absence and presence of the respective inducers. To assess the influence of the intracellular plasmid copy number, $P_{LtetO-1}$ and $P_{lac/ara-1}$ were inserted in plasmids of the pZ-family containing the replication origin of plasmids ColE1, p15A and pSC101*, respectively. The host strain in all experiments was DH5αZ1. The most highly repressed state and the largest regulation factor exceeding a 5000-fold range was found with $P_{LtetO-1}$ when carried on a low copy number plasmid (Table 1a). Higher intracellular plasmid numbers increased the luciferase activity accordingly (7-fold for p15A and 15-fold for ColE1). The luciferase activities in the repressed state did, however, correlate only qualitatively with the copy number. Both $P_{LlacO-1}$ and $P_{A1lacO-1}$ are repressed to about the same level. However, since upon induction $P_{LlacO-1}$ produces twice the amount of luciferase, its regulation factor is higher (620- versus 350-fold). Examining $P_{lac/ara-1}$, a regulatory range of 1700–1800-fold is found irrespective of the intracellular plasmid copy number which, nevertheless, affects the absolute values of repression and induction (Table 1b). The lowest luciferase activity in the repressed state was again observed with plasmids of the pZS* series as expected. For all promoters, the activity in the fully induced state was identical to their activity in the repressor-free strain DH5α (data not shown).

The potential to quantitatively control a gene activity with the promoters described is exemplified by experiments depicted in Figure 4. The luciferase gene as well as the gene encoding the low abundance *E.coli* chaperone DnaJ were placed under the control of $P_{LtetO-1}$, $P_{LlacO-1}$ and $P_{lac/ara-1}$, respectively and the activity of the promoters was analyzed at various concentrations of inducers. The dose response curves show that partial induction can be achieved with all promoters and that $P_{lac/ara-1}$ can be tuned particularly well since induction with IPTG and activation with arabinose allows a high degree of differentiation. The induction curve of promoter $P_{LtetO-1}$ suggests a strong cooperative effect in the binding of the inducer aTc to the *Tet* repressor. The same phenomenon was observed with several other *Tet*R regulated constructs (data not shown). The lower part of Figure 4 shows the controlled expression of DnaJ. Western blots demonstrate that the repressed state is hardly different from the cellular background (~100 DnaJ molecules/cell) (43) whereas full induction yields high levels of expression with all three promoters.

Cloning and expression of a gene encoding restriction endonuclease *Cfr*91

Based on earlier results (44,45) it can be estimated that under repression conditions promoters like $P_{LtetO-1}$ and $P_{lac/ara-1}$ when placed on a low copy number plasmid such as pZS* produce less than one mRNA per cell. This should permit the cloning of genes encoding highly toxic products. To test this prediction, the gene of the *Cfr*9I restriction endonuclease was cloned in absence of its cognate methyltransferase. The coding sequence of *Cfr*9I was placed under the control of $P_{lac/ara-1}$ in plasmids pZS*24ΔRBS, pZA24ΔRBS (where RBSII was deleted) and pZA24. In all three plasmids, the gene could be stably maintained in DH5αZ1 and growth rates of cells harbouring pZS*24ΔRBS-cfr were indistinguishable from cells without any plasmid (Fig. 5a). However, cells containing pZA24-cfr formed colonies with a mucoid phenotype. Induction of transcription by IPTG or by IPTG and arabinose led to immediate growth arrest of the culture (Fig. 5b and c). Since in *E.coli* protein synthesis can continue for some time after the destruction of chromosomal DNA (46) the feasibility of producing *Cfr*9I endonuclease in DH5αZ1 was examined. Indeed using pZA24-cfr, the endonuclease could be produced to a level corresponding to ~2% of the total cellular protein, despite immediate growth arrest of the culture upon induction (Fig. 5d).

DISCUSSION

The transcription control systems described here expand our capabilities of studying gene function *in vivo*. First, gene activities can be regulated over a wide range spanning more than three orders of magnitude but more importantly they can be repressed extremely tightly. This opens up the possibility of varying the concentrations of regulatory proteins which, under physiological conditions, are present at very low levels. Examples for such proteins may be the central heat shock regulator of *E.coli* σ^{32}, the chaperone DnaJ or ftsZ, a crucial component in the signalling pathway of cell division. Second, by exploiting the three regulatory principles, *Lac*R/O, *Tet*R/O and AraC/I_1-I_2, several gene activities can be independently regulated. This will allow the analysis of intracellular equilibria by varying the concentrations of participants and elucidate their contribution to a phenotype.

The crucial developments for the expression system described here were the promoter–operator combinations which were conceived following principles described earlier (12,15). Accordingly, promoters were selected which exhibit low or intermediate rates of complex formation with RNA polymerase. Moreover, operators were positioned in regions shown to be most effective. Thus, provided a 17–19 bp operator sequence binds a repressor sufficiently tightly, it can be accommodated in the spacer region of a promoter where it interferes with RNA polymerase binding most efficiently (15) and where it perturbs least the functional

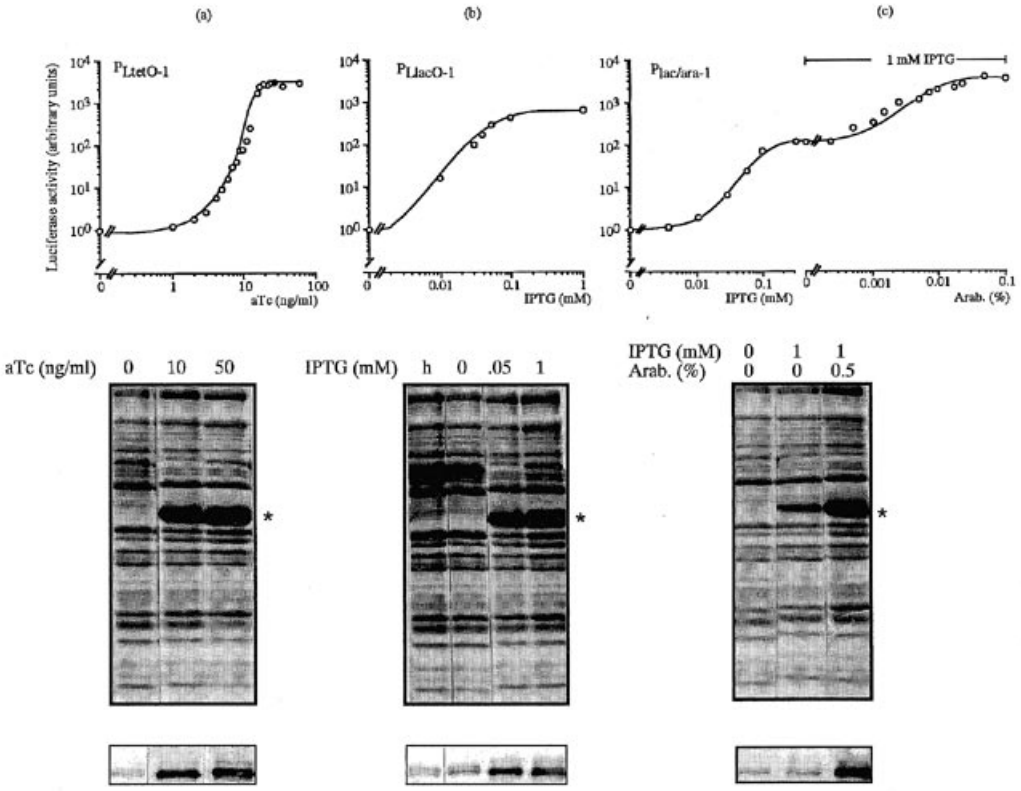


Figure 4. Regulation of the activity of $P_{LtetO-1}$, $P_{LlacO-1}$ and $P_{lac/ara-1}$. All promoters were inserted in pZE2 plasmids where they directed the synthesis of luciferase or DnaJ, respectively. The resulting plasmids (pZE21-luc, pZE21-dnaJ; pZE22-luc, pZE22-dnaJ; pZE24-luc, pZE24-dnaJ) were transferred into *E.coli* DH5αZ1 and luciferase activity as well as DnaJ synthesis were monitored at different states of induction. The upper part in all three panels shows a dose response curve depicting the luciferase activity in dependence of the inducer. In the part below, Coomassie stained polyacrylamide gels (12%) obtained after electrophoresis of total cellular protein show the synthesis of DnaJ (asterisk) under the conditions indicated. The lowest panel depicts Western blots obtained from such gels (1/50 of the protein applied) with anti DnaJ antibodies. (**a**) Induction of luciferase activity and DnaJ under the control of $P_{LtetO-1}$ at aTc concentrations indicated. (**b**) Same as in (a) but controlled by $P_{LlacO-1}$ and IPTG. (**c**) Control of luciferase and DnaJ synthesis by $P_{lac/ara-1}$. The differential regulation by IPTG and by IPTG and L(+)-arabinose is shown. Lane h in (b) denotes a protein extract of plasmid free host cells. For unknown reasons the electrophoretic pattern of DnaJ occasionally exhibits a double band with varying stoichiometry.

program of a promoter. The second best choice for placing an operator is position III where the *lac* operator sequence, however, diminishes promoter clearance by RNA polymerase (22).

For the first class of regulatable promoters, P_L of phage lambda served as a paradigm. It is a strong and highly repressible promoter *in vivo* which, however, binds RNA polymerase with a moderate forward rate constant. By combining this promoter with *tet* operators, $P_{LtetO-1}$ was obtained whose activity can be controlled via *Tet*R and anhydrotetracycline. It is a strong promoter *in vivo* and can, nevertheless, be repressed up to 5000-fold in *E.coli* DH5αZ1. This is the widest range of regulation measured for any *E.coli* promoter so far using the Luciferase reporter system. Partial induction of $P_{LtetO-1}$ is achieved by varying the concentration of aTc (Fig. 4a). In contrast to tetracycline, anhydrotetracycline is a particularly useful inducer. It binds *Tet*R with an ~35-fold higher binding constant and thus allows to operate at very low concentrations. At the same time, its antibiotic activity is ~100-fold lower (47) and concentrations of <50 ng/ml as required for the full induction of $P_{LtetO-1}$ have no effect on the growth of *E.coli*. The finding that repression is less effective at higher plasmid copy numbers may be due to the different ratio of operators to repressors as well as to the increase in unspecific binding sites which affects the concentration of free repressor. Following the same strategy but using the *lac* operator sequences, $P_{LlacO-1}$ was constructed. It is a strong promoter which can be regulated over a >600-fold range. From the results shown in Table 1, we anticipate that placing this promoter into low copy number plasmids, it will permit a similar tight repression of transcription as $P_{LtetO-1}$ and will therefore also be suitable for controlling gene products at very low intracellular levels. Promoter $P_{A1lacO-1}$ contains one of its *lac* operators in position III (Fig. 1) which limits the rate of promoter clearance by RNA polymerase. Thus, it is a somewhat weaker promoter which, nevertheless, is well regulatable (Table 1a).

While P_L is an example for a highly repressible, strong promoter with a moderate k_{ON}, P_{lac} is an example for a promoter whose high repressibility is due to its low rate of polymerase binding. This, however, limits its activity in the derepressed state. For full activity it requires the upstream binding of CRP/cAMP. But even when fully activated, P_{lac} remains a moderately strong promoter. Examining a number of P_{lac} mutants, P_{lac-8A} exhibited interesting features: its *in vivo* strength when derepressed but not activated was 16 times but its k_{ON} only three times higher than that of P_{lac}. It also could still be activated by CRP/cAMP. The repressibility of this promoter was optimized by introducing a symmetrical 20 bp *lac*O sequence into the spacer region (overlapping with the –10 and the –35 hexamer by 1 bp each) and by placing a third operator at position VI (Fig. 1). To avoid pleiotropic effects by CRP/cAMP activation, the AraC binding site I_1-I_2 of the *ara* BAD promoter replaced the CRP/cAMP site. The resulting

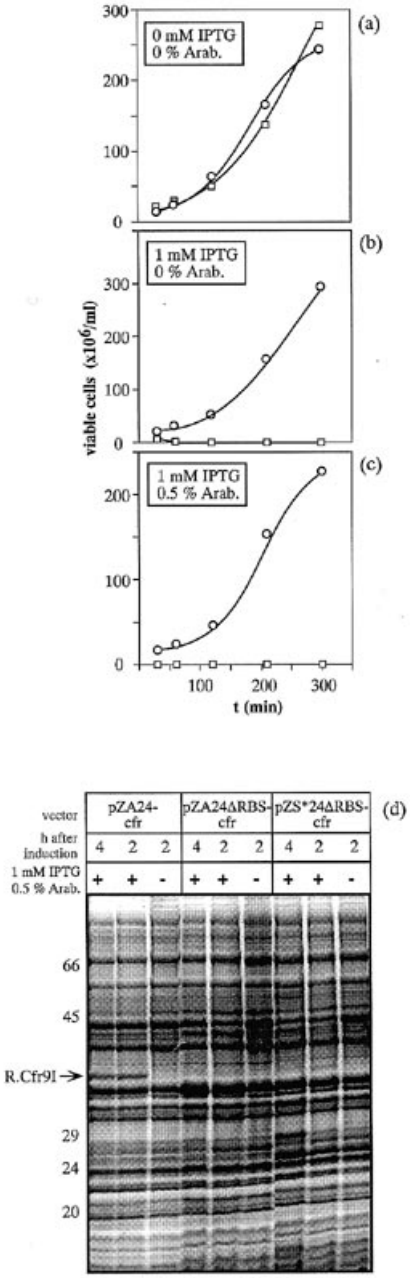


Figure 5. (**a**) Controlling the gene encoding the restriction endonuclease *Cfr*9I. Overnight cultures of *E.coli* DH5αZ1 or *E.coli* DH5αZ1 harbouring pZS*24ΔRBS-cfr were diluted 1:100 and grown at 37°C in absence of IPTG and L(+)-arabinose (a), in presence of IPTG only (b) and in presence of IPTG and L(+)-arabinose (c) as indicated. IPTG or IPTG and L(+)-arabinose were added at time zero and aliquots of cells (diluted 1:1000) were plated on LB-Sp[50 µg|ml] plates at the times indicated. After 16 h, the number of colonies was determined. Circles depict the control culture of DH5αZ1, squares show cultures of plasmid containing cells. (**d**) Electrophoretic analysis of cell extracts after induction of *Cfr*9I synthesis. DH5αZ1 cells harbouring pZA24-cfr, pZA24ΔRBS-cfr and pZS*24ΔRBS-cfr, respectively, were grown to OD_{600} = 0.5 before $P_{lac/ara-1}$ was induced by IPTG and L(+)-arabinose as indicated. Cells were harvested after 2 and 4 h and proteins were electrophoretically separated in a 12% SDS polyacrylamide gel. The position of the restriction endonuclease is indicated.

promoter ($P_{lac/ara-1}$) is regulatable over an ~1800-fold range and when fully induced and activated it exceeds the *in vivo* strength of P_{lac} 6-fold. Thus, it is a strong and highly regulatable promoter. The fine tuning of $P_{lac/ara-1}$ is facilitated by a two step mechanism: increasing the IPTG concentration in the medium up to 0.2 mM leads to an ~100-fold induction which can be enhanced 15–20-fold by adding arabinose to a final concentration of 0.03% (Fig. 4c).

Addition of glucose (0.6%) to the growth medium decreased the activation potential of AraC 2–3-fold (data not shown). This is most likely due to the reduction of *araC* transcription which is controlled by CRP/cAMP (48). This glucose effect can of course be avoided by replacing glucose with glycerol or other non-PTS sugars as a primary carbon source when, for example, minimal medium is required for culturing.

Repression and induction depend on a number of parameters such as the concentration of free repressor and the increment by which an inducer decreases the affinity of a repressor to its operator. Free repressor concentration is also a function of the number of unspecific (and specific) DNA binding sites and may thus be affected by plasmid copy number and size although this is a minor parameter with the plasmids described herein. A simple increase of the intracellular repressor concentration on the other hand does not necessarily compensate for this effect since the residual affinity of the repressor–inducer complex to the respective operator sequence prevents full induction as seen for both TetR and LacR (data not shown). Moreover, high repressor concentrations may be toxic for the cell as is the case for TetR (ref. 49 and our unpublished results). Incomplete induction is frequently encountered with the widely used tac or trc type promoter systems because these high 'k_{ON}' promoters are reasonably well repressed only at very high intracellular repressor concentrations. When examined under conditions as defined in Table 1 repression of these promoters is only 10–20-fold (data not shown). It is therefore important to establish stable intracellular conditions where the relevant regulatory proteins are present in defined concentrations which warrant a reliable control of promoters under various physiological conditions. This was achieved by integrating the *laci* as well as the *tetR* gene controlled by promoters of appropriate *in vivo* strength into the *E.coli* chromosome. The high 'k_{ON}' constitutive promoters P_iq and P_{N25} ensure efficient transcription even under conditions of reduced concentration of active σ^{70} RNA polymerase e.g. in stationary phase. The resulting *E.coli* strains DH5αZ1 and W3110Z1 produce constitutively around 3000 tetrameric *Lac* and 7000 dimeric *Tet* repressors per cell during logarithmic growth. Sufficient AraC is supplied by its natural autoregulated pathway as its overproduction from a plasmid did not lead to increased activation of $P_{lac/ara-1}$ (data not shown). Thus, *E.coli* strains of the DH5αZ1 type provide all regulatory proteins required in appropriate amounts for tight repression and full induction (which is indistinguishable from repressor-free host strains; data not shown) at different plasmid copy numbers. The tight repression is maintained also in stationary phase and in overnight cultures (data not shown). The placement of repressor encoding units onto the chromosome has also simplified the vector constructs and increased the degree of freedom of the system.

Although the regulatory range of the promoters described is large, it may not satisfy all needs. For example for the tight control of a low abundance or toxic gene product, even the fully repressed $P_{LtetO-1}$ may generate a too high background when contained in a ColE1-type plasmid. The vector system therefore offers still another degree of freedom. By utilizing different origins of replication, the intracellular number of plasmids can be varied between ~4 and 60, which permits to shift the regulatory window

of a promoter within an ~15-fold range. Thus, by fully exploiting the potential of the system using, for example $P_{LtetO-1}$, a gene's activity can be controlled over an ~60 000-fold range. The controlled synthesis of a restriction endonuclease, a low abundance *E.coli* protein and luciferase under different conditions as exemplified in Figures 4 and 5 illustrates some of these aspects. Needless to say that three of the replication origins adjusted to fit the vector system belong to different plasmid compatibility groups and thus permit to maintain two or even three vectors within DH5αZ1 cells if required.

It may be of interest to speculate on the absolute tightness achieved, for example, with $P_{LtetO-1}$ in DH5αZ1 cells. When fully induced, this promoter has an activity of ~30 P_{bla} units (45) and is estimated to initiate transcription ~5-fold less frequently than the fully activated *rrnB*P1 promoter (12). The *rrnB*P1 promoter is estimated to initiate 1.5 mRNAs/s at maximal growth rates during logarithmic growth (44). Hence, it can be estimated that $P_{LtetO-1}$ initiates 0.3 mRNAs/s. Given a generation time of 25 min for *E.coli* in log phase cultures a 5000-fold repression of this promoter would reduce this rate to 6.5×10^{-5} mRNAs/s or in other words one mRNA every 10th generation would be synthesized in a single copy situation. Thus, $P_{LtetO-1}$ located on a plasmid of the pZS*-type giving rise to three to four copies/cell will produce one mRNA about every 3rd generation. The luciferase activity monitored with $P_{LtetO-1}$ in the repressed state which corresponds to an average of 12 enzyme molecules per cell is not in disagreement with these estimates. This suggests that at the repression levels achieved only a fraction of a cell population synthesizes a given gene product at any one time. Populations would therefore survive if this gene product was highly poisonous as for example the restriction enzyme Cfr9I since only a minor portion of cells would die.

The tight control of transcription, the potential to regulate gene activities quantitatively over wide ranges and the possibility to control independently several transcription units in a cell are the main advantages of the system described here when compared to other commonly used promoter/vector combinations. It thus opens up new perspectives for the study of cellular physiology as well as for the controlled expression of heterologous genes.

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