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Short Communication

Design of a Novel Regulatory Circuit for Expression of Restriction Endonucleases

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We have developed a new strategy with a very tight control for the expression of cloned genes. The system employed here is the T7 promoter-based expression system in which transcription activator protein C of bacteriophage Mu (Mu C) has been cloned to serve as a repressor in the regulatory circuit. The system also includes pLysE, which encodes T7 lysozyme, an inhibitor of T7 RNA polymerase. This ensures tight regulation of cloned genes in the uninduced state. Upon induction, the expressed Mu C protein binds to its cognate site thereby repressing *lys* transcription driven by the *tet* promoter. In order to evaluate the tight control achieved in the system, and to check leaky expression, if any, we have cloned the gene for the *SmaI* restriction endonuclease without its cognate methylase. For this purpose, a dicistronic unit was constructed by cloning the *smaIR* gene downstream of the Mu C gene. *SmaI* expression was observed only in the induced cell extracts, demonstrating a tight control. The system could be used to express the genes of other cloned restriction enzymes and has the potential for general applications.

Key words: Artificial operon / Mu C protein / *Sma*I endonuclease / T7 lysozyme / Transcription terminator / T7 RNA polymerase.

Restriction endonucleases are invaluable tools in recombinant DNA technology and in the study of protein-DNA interactions by virtue of their exquisite specificity. Restriction endonucleases are usually accompanied by cognate methylases. Genes encoding components of a restriction modification system are usually located in close proximity to each other. About 80 R-M systems have been cloned so far (Bickle and Krüger, 1993; Roberts and Halford, 1993). The cloning of a majority of restriction endonucleases has involved the cloning of the cognate methylase first by the 'Hungarian trick' or the methylase selection method (Mann *et al.*,1978; Szomolanyi *et al.*, 1980; Kiss *et al.*, 1985; Morgan *et al.*, 1996) in order to protect the host DNA from cleavage by the endonuclease. Cloning R-M systems has also been achieved by using a screen involving methyl-directed restriction systems or production of DNA damage on unmethylated DNA by expression of thermostable endonucleases (Piekarowicz *et al.*, 1991; Fomenkov *et al.*, 1994) and methods involving phage restriction (Walder *et al.*, 1981). In all these methods, prior expression of the cognate methylase is necessary for the stable, functional expression of the endonuclease.

We have now developed a new strategy applicable for the expression of cloned restriction endonuclease genes without involving the cognate methylases. For this purpose, a tightly controlled regulatory circuit has been constructed. A T7 promoter-based expression system comprising two components has been used here. The first component of this system is a T7 expression vector, pET11d (Studier *et al.*, 1990). The second component is the vector pLysE, which harbors the T7 lysozyme gene in the expressed orientation. T7 lysozyme is a natural inhibitor of T7 RNA polymerase and inhibits transcription by direct interaction with the polymerase (Zhang and Studier, 1997). This property has been harnessed in controlling leaky expression by T7 RNA polymerase in the system (Studier,1991). The overall control circuit for the regulated expression used for the expression of restriction endonucleases is depicted in Figure 1. As a part of the scheme, the gene for the bacteriophage Mu transcription activator C was cloned in pET11d and its cognate sites in pLysE. Thus, when the Mu C protein is produced, it binds to its sites with a very high affinity (Ramesh *et al.*, 1994) and downregulates *lys* transcription directed by *tet* promoter (data not shown). This leads to a decrease in the levels of T7 lysozyme, which would otherwise reduce the expression of cloned genes in the induced state (Zhang and Studier, 1995). The vector pLysE was used in our method as it yields high levels of T7 lysozyme resulting in a tight control of leaky expression from the T7 promoter in the uninduced state.

The test for the efficacy of the system would be the expression of any restriction endonuclease that cannot be

Table 1 Primers Used in the Study.

Primer 1.	5′ GCTAGCCATGGACTTATTCGAACACGATCC 3′
Primer 2.	5′ CGGGATCCAAAATAACCGGCAGGA 3′
Primer 3.	5′ CCGGGATCCTAACAAGGCAGGGTT 3′
Primer 4.	5′ GCGGATCCTCTTGCCAAAGAGAGAATAT 3′

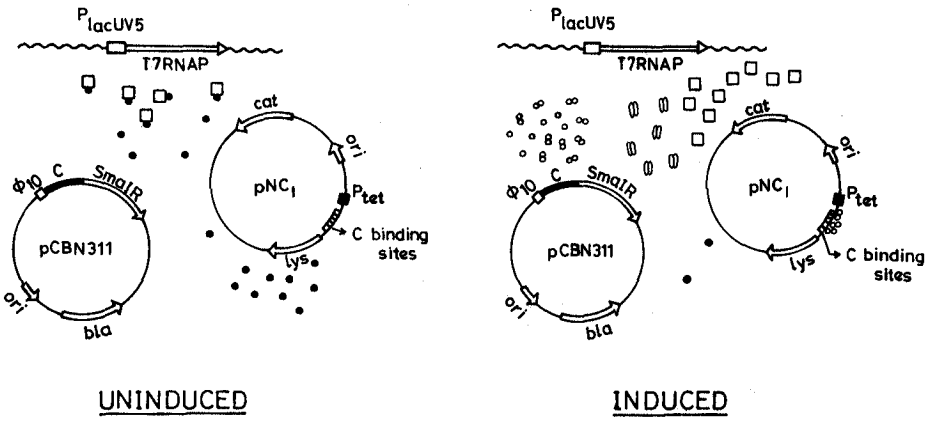

Fig. 1 Control Circuit for Expression of Restriction Endonucleases.
The T7 lysozyme (solid circles) produced from the vector pNC1 binds to T7 RNA polymerase (open squares) in the uninduced state and prevents transcription. In the induced state, T7 RNA polymerase directs transcription from the ϕ_{10} promoter. The C protein (open circles) and the *Sma*I restriction endonuclease (open ellipses) are produced. C binds to its cognate sites and represses *lys* transcription. The levels of T7 lysozyme drop, resulting in increased availability of T7 RNA polymerase for transcription. The dicistronic unit of Mu *C* and *smaIR* (pCBN311) was constructed as follows. The Mu *C* gene was amplified from the plasmid pVR7 (Ramesh *et al.*, 1994) by 30 cycles of polymerase chain reaction (PCR) with *Taq* DNA polymerase, using primers 1 and 2 (Table 1). Primer 2 includes a ρ-independent transcription terminator. The PCR product was cloned into the *Nco*I and *Bam*HI sites of pET11d to generate pBN31. The *smaIR* gene was also amplified by PCR using primers 3 and 4 (Table 1) from the genomic DNA of *Serratia marcescens*. The fragment was cloned in the *Bam*HI site of pBN31 downstream of the Mu *C* gene to generate pCBN311. The sequences of the cloned PCR products were confirmed by sequencing.

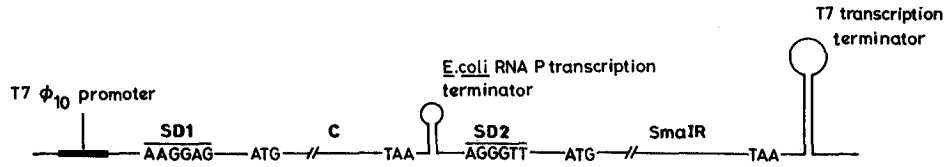

Fig. 2 Organization of the Operon Encoding Mu C and the *Sma*I Restriction Endonuclease in the Plasmid pCBN311.
The Shine-Dalgarno sequences (SD1 for the Mu *C* gene and SD2 for *smaIR* gene) and the start and stop codons of the two genes are indicated. The T7 promoter, T7 transcription terminator and the *E. coli* ρ-independent terminators are depicted.

otherwise expressed in the absence of its cognate methylase. We have chosen the *Sma*I endonuclease of *Serratia marcescens* for this purpose. In the genomic context, *Sma*I R-M genes are transcribed from convergent promoters (Heidman *et al.*, 1989). In addition, there is a small open reading frame that encodes *Sma*I C, which is involved in the regulation of the *Sma*I restriction endonuclease gene (Ives *et al.*, 1995; Tao *et al.*, 1991). We have cloned the gene for the *Sma*I endonuclease alone in our system without the *Sma*I C ORF or the *Sma*I methylase gene. The regulatory scheme is depicted in Figure 1. A pentamer of the Mu C-binding site was cloned in pLysE to generate pNC1. Presence of multiple high-affinity binding sites would ensure efficient repression of the *lys* gene. In our earlier studies we had used a single site to repress *lys* transcription from pLysS, where lower amounts of lysozyme is produced (Paul *et al.*, 1997). The details of the dicistronic expression unit having the proximal Mu *C* gene and the distal *smaIR* gene is described in the legends to Figure 1. The Mu *C* gene was cloned in the vector pET11d to generate pBN31, following which a promoterless fragment amplified from the *Serratia marcescens* genome bearing the *smaIR* gene was cloned downstream of the Mu *C* gene to generate pCBN311 (Figure 1). The overall organization of the regulatory elements in this artificial operon is depicted in Figure 2. The T7 RNA polymerase-specific promoter ϕ_{10} directs the transcription of both the genes. A ρ-independent transcription terminator was introduced between the two cistrons as an additional, yet important, regulatory measure. The transcription terminator prevents any leaky expression originating from weak promoters located either within the Mu *C* gene or elsewhere in the vector. The presence of the second stronger T7-specific terminator at the end of the *smaIR* gene brings about efficient recycling of T7 RNA polymerase. The construct harboring the artificial operon, pCBN311, was transformed into *Escherichia coli* BL26(DE3) alone. No transformants were obtained in this case, reflecting the toxicity of this gene product. This indicates a requirement for either a tight repression of the system or the cognate methylase. In contrast, a large number of colonies were obtained in *E. coli* BL26(DE3) harboring either pNC1 or pACMSma, a pACYC184 derivative harboring the *Sma*I methylase gene, *smaI*M. Cells harboring these constructs were grown to an optical density of 0.6 (600 nm) and induced with 0.3 mM isopropyl β-D-galactopyranoside (IPTG) for 2 h. Cell extracts from the various cultures were assayed for *Sma*I endonuclease activity (Figure 3). *Sma*I restriction

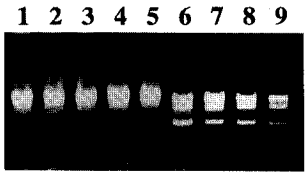

Fig. 3 Activity Assay with Cell Extracts of Cultures Harboring pCBN311.

Escherichia coli BL26(DE3) cells harboring pNC1 were transformed with pCBN311 and transformants were grown in 6 ml LB at 37 °C until an optical density of 0.6 (600 nm). The culture was split into two, and one-half was induced with 0.3 mM IPTG for 2 h. Cells were harvested and sonicated after suspending in 0.5 ml buffer consisting of 10 mM potassium phosphate, pH 7.4, 0.1 mM Ethylenediaminetetra acetic acid (EDTA); 7 mM β-mercaptoethanol and 1 mM Phenylmethylsulfonyl fluoride (PMSF). 5 μl of the uninduced and induced cell extracts were incubated with 1 μg of lambda DNA and incubated at 25 °C for 1 hour and then electrophoresed on a 1% agarose gel (lanes 5 and 6). Lane 1 is uncut DNA. Assays were also performed with induced host cell extracts (lane 2), host harboring either pNC1 or pNC1 and pBN31 (lanes 3 and 4 respectively), and with uninduced and induced cell extracts of host harboring pACMSma and pCBN311 (lanes 7 and 8 respectively). Lane 9 shows activity with commercially available *Sma*I restriction enzyme (New England Biolabs).

endonuclease activity was seen in both the uninduced as well as induced state of *E.coli* BL26(DE3) cells harboring pACMSma, whereas cell extracts from cultures harboring pNC1 exhibited activity only in the induced state. The level of expression of the *Sma*I restriction endonuclease was estimated by determining the total activity. The value of 2×10^4U/l reflects low-level expression. Thus, while the system meets the criteria for expression of a restriction endonuclease without having the corresponding methylase, engineering overexpression would involve additional manipulation (see later section). Thus, the regulatory system ensures tight control of leaky expression. Even very low level expression of an endonuclease could prove detrimental to cell survival as demonstrated earlier for the *Eco*RI restriction endonuclease (Heitman *et al.*, 1989). Cloning of certain R-M systems has posed problems even when the methylase gene was cloned first. Deletions spanning the gene for restriction endonucleases have been encountered in spite of the fact that the R and M genes were contained on a single DNA fragment (Hammond *et al.*, 1990). This necessitated prior modification of the host genome before the introduction of the R-M system. Such multi-step procedures have been resorted to in the case of *Bam*HI (Brooks *et al.*, 1989), *Dde*I (Howard *et al.*, 1986) and *Bsp*6I (Lubys and Janulaitis, 1995). Thus, the regulatory circuit described here offers an attractive alternative for the expression of restriction endonucleases in the absence of their cognate methylases. Moreover, the presence of a strong ρ-independent terminator facilitates initial screening for recombinants without involving the cognate methylase in a strain that does not harbor the gene for T7 RNA polymerase. The successful expression of the *Sma*I restriction endonuclease demonstrates that it should be possible to functionally maintain and stably express genes whose products affect cell survival. With the present approach, the levels of expression of the second gene, *smaIR*, were not high. This could be due to the poor Shine-Dalgarno sequence of the *smaIR* gene. Additionally, the *E. coli* terminator might be strong enough to terminate T7 RNA polymerase-directed transcription at the end of the Mu *C* gene. There have been reports discussing the ability of certain *E. coli* terminators to function as T7 transcription terminators (Christiansen, 1988; Jeng *et al.*, 1990; Macdonald *et al.*, 1993; Lyakho *et al.*, 1997). It should be noted here that the present study was directed at regulated expression without bringing in the cognate methylase and not designed for overexpression. The system could be engineered for high-level expression with appropriate alterations in the regulatory system. One approach is to introduce a strong Shine- Dalgarno sequence for the *R* gene at an optimal distance from the start codon. Tinkering with the structure of the ρ-independent transcription terminator to ensure efficient transcription of the second gene of the operon would be another strategy.

Several other strategies have been developed to provide very tight control. These include the use of antisense RNA complementary to the mRNA of the gene of interest (O'Connor and Timmis, 1987), use of the antitermination function of the phage λ-derived nutL/N protein (Mertens *et al.*, 1995) or promoter inversion mediated by recombination (Hasan and Szybalski, 1987). While these methods have their own merits and have been designed for a specific purpose, the novel feature of our method is the construction of an artificial operon for regulated expression. The components (Mu C protein on one plasmid and its binding sites on another plasmid) could be used for the expression of other restriction endonucleases. The system can prove especially useful where the methylase is poorly expressed or has not been identified.

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