Selective Overproduction of Chloramphenicol Acetyltransferase in the T7 Expression System

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T7 발현체계에서 Chloramphenicol Acetyltransferase 의 선택적 과잉생산

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ABSTRACT: A gene can be selectively overexpressed in *E. coli* by utilizing the phage T7 RNA polymerase's stringent recognition and active transcription of the T7 promoter. The T7 expression system was constructed such that the T7 RNA polymerase gene is under the control of *lac*UV5 promoter in one plasmid, and that the target gene, the promoterless chloramphenicol acetyltransferase (CAT) gene with *E. coli* ribosome binding site is under the control of T7 promoter in the other plasmid. Only the *E. coli* cells containing both plasmids show high resistance to chloramphenicol. When the copy number of the runaway plasmid containing the polymerase gene was varied by a temperature shift, amounts of the CAT protein synthesized upon induction was correspondingly changed as shown in SDS gel electrophoresis.

KEY WORDS □ T7 RNA polymerase, T7 promoter, chloramphenicol acetyltransferase, runaway plasmid

T7 RNA polymerase, the product of gene 1 of phage T7 is a monomeric enzyme with a molecular weight of about 100,000. It is specific for its own unique promoters that are 17 bp long in an undisrupted stretch and are not utilized by *E. coli* RNA polymerase. It is capable of efficient RNA synthesis from almost any DNA (Studier and Moffatt, 1986).

The T7 RNA polymerae gene, gene 1 has been cloned by Studier (Davanloo *et al.*, 1984). Since T7 RNA polymerase is very active and selective, the T7 expression system has been developed and this made selective overproduction of specific RNAs and proteins possible (Tabor and Richardson, 1985; Rosenberg *et al.*, 1987).

Here we constructed a T7 expression system to study the effects of T7 RNA polymerase level on expression. Gene 1 is under the control of the inducible *lac*UV5 promoter in one plasmid, and the target gene, the chloramphenicol acetyltransferase (CAT) gene is under the control of a T7 promoter in the other plasmid. Before induction the T7 expression system is repressed by the *lac*I repressor. T7 RNA polymerase induced by IPTG is capable of specific and efficient transcription of the CAT gene. To study the effects of various levels of T7 RNA polymerase on the CAT expression, different levels of T7 RNA polymerase were established. One way to achieve the goal is to use a runaway plasmid (Uhlin *et al.*, 1979). Its copy number can be controlled by a temperature shift, as its replication is regulated by a temperature-sensitive mutant repressor. With increasing temperatures, copy number increases. Thus, it was possible to study

the effects of different copy numbers of gene 1 on the CAT expression, which was measured by SDS-PAGE and chloramphenicol resistance on plates.

MATERIALS AND METHODS

Bacteria and plasmids

The strains BL21, BL21 (DE3) were from W. Studier (Studier and Moffatt, 1986). BL21 is devoid of the OmpT and the Lon proteases (Groberg and Dunn, 1988). BL21 (DE3) carries in the chromosome gene 1 from phage T7 under the inducible *lac*UV5 promoter that can be derepressed by IPTG. The pAR1219 plasmid also carries gene 1 and *lac*I (Davanloo *et al.*, 1984). The construction of pBK and pET3CAT is described in RESULTS AND DISCUSSION. BL21/pET3CAT and BL21 (DE3)/pET3CAT were constructed by transforming BL21 and BL21 (DE3) with pET3CAT. Construction of BL21/pBK/pET3CAT was done by cotransformation of BL21 with pBK and pET3CAT.

Cell growth

A single colony of BL21/pET3CAT or BL21 (DE3)/pET3CAT was inoculated into Luria broth (LB) medium containing 200 $\mu g/ml$ ampicillin. A single colony of BL21/pBK/pET3CAT was also inoculated into LB medium containing 50 $\mu g/ml$ ampicillin and 50 $\mu g/ml$ kanamycin. Growth was allowed at 37 and 30°C. T7 RNA polymerase was induced with 0.5 mM IPTG when the culture reached A_{600} of 0.4, and culture was continued for another 3 h.

Quantitation of chloramphenicol acetyltransferase

Synthesis of chloramphenicol acetyltransferase (CAT) was measured by chloramphenicol resistance on agar plates containing different concentrations of chloramphenicol. Since some overexpressed proteins are inactive when purified *in vitro*, for instance forming inclusion bodies, overexpressed CAT was also measured by SDS-PAGE, rather than by enzyme activity assay. All cultures induced with IPTG and uninduced ones were adjusted to A_{600} of 0.7, pelleted, resuspended in 100 μl of Laemmli sample buffer and boiled for 5 min, and 10 μl of each preparation was analyzed by SDS-13% PAGE, which was then stained with Coomassie brilliant blue R-250.

RESULTS AND DISCUSSION

Construction of plasmids pBK and pET3CAT

Plasmid pAR1219 carrying gene 1 (Davanloo *et al.*, 1984) and runaway plasmid pSY343 (Yasuda and Takagi, 1983) were cut with *Bam*HI and ligated to insert the fragment carrying gene 1 and the *lac*I gene into the *Bam*HI site of pSY343. After ligation mixture was transformed into BL21, kanamycin-resistant and ampicillin-sensitive colonies were isolated and analyzed for inserts. The plasmid carrying gene 1, *lac*I gene and pSY343 replication origin was named pBK (Fig. 1). The copy number of pBK can be controlled by a temperature shift as it contains the runaway replication origin of pSY343.

The pET3CAT plasmid was constructed as shown in Fig. 2 by inserting the *Bam*HI fragment of pJS133 into the *Bam*HI site of pET-3 (Rosenberg *et al.*, 1987). The *Bam*HI fragment of

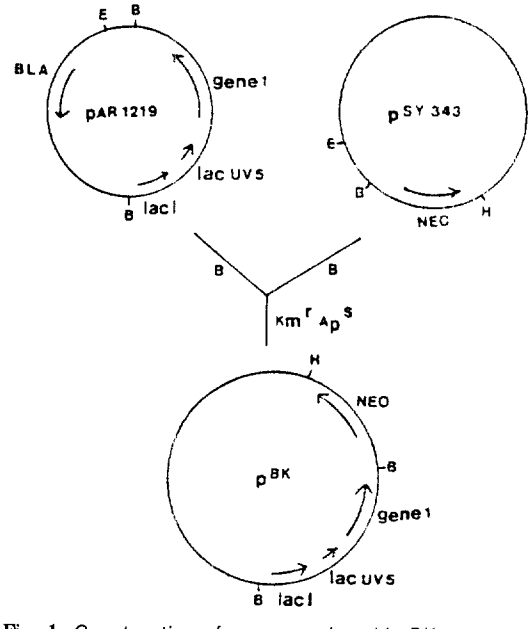

Fig. 1. *Construction of runaway plasmid pBK.*
The *Bam*HI fragment containing *lac*I gene, *lac*UV5 promoter and gene 1 (the T7 RNA polymerase gene) of pAR1219 was inserted into the unique *Bam*HI site of pSY343. Ampicillin (Ap) resistant gene (BLA) and kanamycin (Km) resistant gene (NEO) were used for selection of the plasmids. *Bam*HI (B), *Eco*RI (E), and *Hin*dIII (H) restriction enzyme sites are shown.

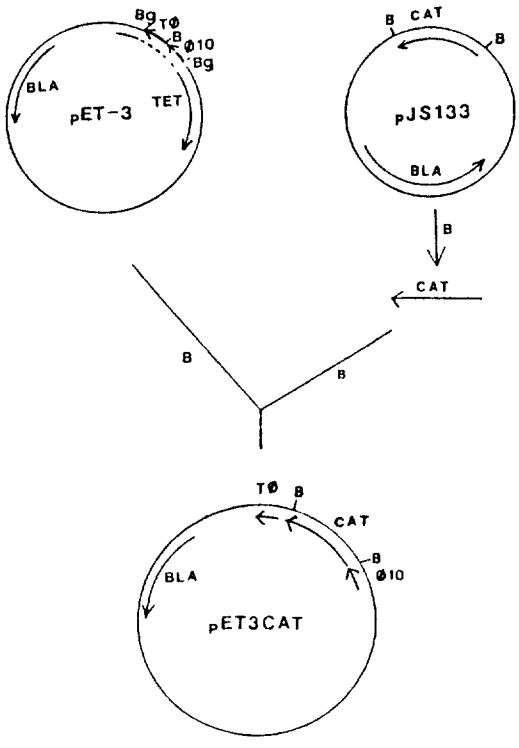

Fig. 2. *Construction of pET3CAT.*
The smaller *Bam*HI fragment containing chloramphenicol acetyltransferase gene (CAT) of pJS133 was inserted into the unique *Bam*HI site of pET-3, which is located between the T7 promoter $\phi 10$ and the T7 terminator $T\phi$. Tetracycline resistant gene (TET) was destroyed by inserting the T7 promoter and terminator. *Bam*HI (B) and *Bgl*II (Bg) restriction enzyme sites are shown.

pJS133 carries the promoterless CAT gene with *E. coli* ribosome binding site sequence and at its upstream the translational stop codons in all three reading frames (Shiau and Smith, 1988). The *Bam*HI site of pET-3 is located between the T7 Ø10 promoter and its terminator TØ. In this construct, the CAT expression of pET3CAT is only possible by transcriptional activity of T7 RNA polymerase.

Production of T7 RNA polymerase

BL21 (DE3) carries one copy of gene 1 in the chromosome and T7 RNA polymerase can be produced by IPTG induction. The pBK plasmid carries *lac*I gene, pSY343 replication origin and gene 1. The copy number of gene 1 of pBK can be regulated just by a temperature shift, since gene 1 is on runaway plasmid pSY343.

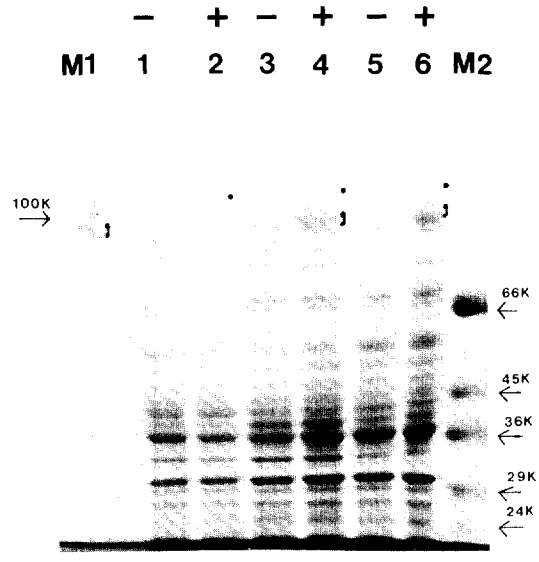

Fig. 3. *Accumulation of T7 RNA polymerase.*
Extracts of BL21(DE3) cultured at 37 °C (lanes 1,2), and those of BL21/pBK cultured at 30 °C (lanes 3,4) and 37 °C (lanes 5, 6) were analyzed by SDS-PAGE, with size markers in lane M2. Cultures were either uninduced (–) or induced (+) with IPTG. The position of β-galactosidase synthesized upon IPTG induction is marked by a dot in lanes 2,4,6 and that of T7 RNA polymerase by a bracket in lanes M1, 4, 6.

The induced level of T7 RNA polymerase is barely detectable in BL21 (DE3) that carries gene 1 in the chromosome, but BL21/pBK induced at 30 and 37 °C produced detectable amounts of T7 RNA polymerase (Fig. 3). The different levels of T7 RNA polymerase between BL21 (DE3) and BL21/pBK are attributed to the fact that BL21 (DE3) has one copy of gene 1 and BL21/pBK has many copies. The β-galactosidase protein was also expressed from the *lac*Z gene in the chromosome as shown in Fig. 3.

CAT synthesis without IPTG induction

In pET3CAT the CAT gene is under the control of a T7 promoter. *E. coli* RNA polymerase hardly recognizes the T7 promoter. BL21/pET3-CAT that does not contain gene 1 expressed very low level of CAT probably by *E. coli* RNA polymerase, considering that BL21/pET3CAT grew on plates containing $2\mu g/ml$ chloramphenicol, but did not grow on those containing $5\mu g/ml$ (Table 1).

Significant CAT activity was detected in BL21

Table 1. *Chloramphenicol resistance on plates*

Strain	Culture temp. (°C)	Chloramphenicol (μg/ml)			
		2	5	15	15+IPTG
BL21	37	–	–	–	–
BL21(DE3)	37	–	–	–	–
BL21/pET3CAT	37	+	–	–	–
BL21(DE3)/pET3CAT	37	+	+	+	–
BL21/pBK/pET3CAT	37 or 30	+	+	+	–

+ indicates colony formation, and – no growth. Concentration of IPTG is 0.5 mM.

(DE3)/pET3CAT that carries gene 1 in the chromosome even without IPTG induction. BL21 (DE3)/pET3CAT could grow on plates with 15 μg /ml chloramphenicol. The CAT expression without IPTG induction was higher in BL21 (DE3)/pET3CAT than in BL21/pET3CAT. Without induction, the *lac*UV5 promoter in the upstream of gene 1 is repressed by the *lac*I gene. Since repression is incomplete, some T7 RNA polymerases are produced even without induction. Most of the CAT expression in BL21 (DE3)/pET3CAT is achieved by the residual level of T7 RNA polymerase.

The pBK and pET3CAT plasmids can coexist in a cell, since their replication origins belong to different incompatibility groups. BL21/pBK/pET3CAT could grow on plates containing 70 μg/ml chloramphenicol. The results show that most of CAT are expressed in BL21/pBK/pET3CAT not by *E. coli* RNA polymerase but by T7 RNA polymerase even in the absence of induction.

CAT synthesis with IPTG induction

When BL21 (DE3)/pET3CAT and BL21/pBK/pET3CAT were induced by IPTG at A_{600} of 0.4, CAT were expressed to a high level and synthesis of host proteins was reduced as shown by SDS-PAGE in Fig. 4.

When induced, at the beginning T7 RNA polymerase under the *lac*UV5 promoter will be produced by *E. coli* RNA polymerase that initiates transcription at the *lac*UV5 promoter derepressed by IPTG. Then T7 RNA polymerase will recognize the T7 promoter and transcribe the CAT gene specifically and efficiently. With time T7 RNA polymerase will compete with *E. coli* RNA polymerase in transcription for precursors in the presence of IPTG. Since elongation of RNA chains

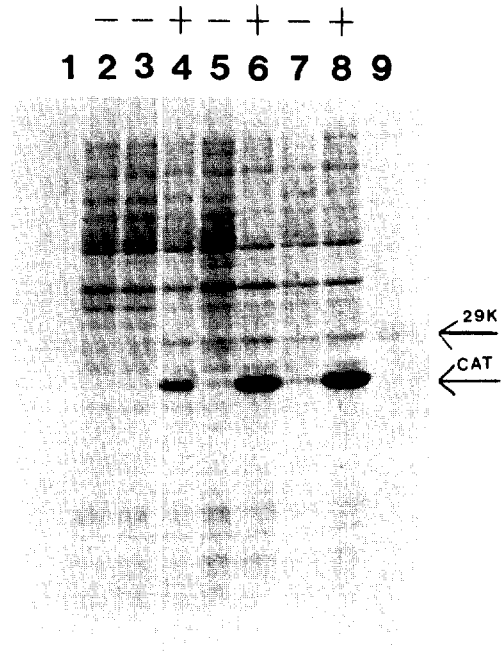

Fig. 4. *CAT synthesis without and with IPTG induction.* Lysates of BL21(DE3)/pET3CAT cultured at 37 °C (lanes 3,4), and those of BL21/pBK/pET3CAT at 30 °C (lanes 5,6) and at 37 °C (lanes 7,8) were analyzed by SDS-PAGE. Lysates of BL21/pET3CAT cultured at 37 °C (lane 2) were run as a control. The size marker protein in lanes 1 and 9 is an *E. coli* ribose binding protein (29 Kd). + indicates IPTG induction, and – no induction.

by T7 RNA polymerase is about 5 times faster than that by *E. coli* RNA polymerase, T7 RNA polymerase outcompetes the host enzyme and inhibits transcription by *E. coli* RNA polymerase. As gene 1 is under the *lac*UV5 promoter that is utiliz-

ed by *E. coli* RNA polymerase, synthesis of the T7 RNA polymerase will reach a steady state.

Studier and Moffatt (1986) proposed that delivering the target gene by phage infection, after accumulation of T7 RNA polymerase in the cell to the desired level, would make study of the effects of various levels of T7 RNA polymerase possible. In studying the effects of copy number of gene 1, however, the limitation can be overcome by using a runaway plasmid, pBK.

A runaway plasmid has a moderate number of copies less than a hundred at 30°C, but loses control of replication at 37°C so that amplification of plasmids is achieved (Uhlin *et al.*, 1979). For instance, a temperature shift from 28°C to 42°C increases the plasmid copy number several fold to hundreds of copies (Remaut *et al.*, 1983). There are reports that some proteins are produced two to three fold more at 37°C than 30°C, using a runaway plasmid (Nishimura *et al.*, 1987; Yasuda and Takagi, 1983). At 37°C, the copy number of pBK will be higher than at 30°C and higher amounts of T7 RNA polymerase produced by IPTG induction will result in higher amounts of CAT than at 30°C.

In BL21 (DE3)/pET3CAT containing only one copy of the T7 RNA polymerase gene, gene 1, the amount of CAT produced by IPTG induction was 20% of the total cellular protein content, as determined by densitometric analysis of SDS-PAGE. At 30 and 37°C, CAT produced by IPTG induction in BL21/pBK/pET3CAT containing multiple copies of gene 1 was 30 and 36%, respectively, of the total protein content. Thus, the amount of CAT synthesized was correspondingly changed as shown in SDS-PAGE, as the copy number of gene 1 was varied by a temperature shift.

Test of the T7 expression system and its usefulness

One way to know whether the T7 expression system maintains the target gene is to check the growth of cells on plates containing chloramphenicol and IPTG. BL21 (DE3) carrying a plasmid without a T7 promoter, such as pBR322 makes colonies on plates containing ampicillin plus IPTG. However, BL21 (DE3) carrying a plasmid with a T7 promoter cannot form colonies in the presence of IPTG. BL21 (DE3)/pET3CAT and BL21/pBK/pET3CAT can grow on plates containing 15 μg/m*l* chloramphenicol but not on those of 15 μg/m*l* chloramphenicol plus 0.5 mM IPTG (Table 1). The deleterious effect might be explained by that efficient transcription by T7 RNA polymerase exhausts ribonucleoside triphosphates. The inability to make colonies on plates containing chloramphenicol and IPTG means that the T7 expression system keeps the target gene in the presence of T7 RNA polymerase and the system works properly.

In this study we demonstrated not only selective overproduction of CAT in the T7 system but also quantitative effects of the polymerase gene copy number on the CAT synthesis. Several proteins, whose significant production had been impossible in other gene expression systems, were overproduced in the T7 system owing to the promoter specificity and strong transcriptional activity of T7 RNA polymerase (Toyoda *et al.*, 1986; Rosenberg and Studier, 1987; McKinney *et al.*, 1987; Singer *et al.*, 1989; Williams *et al.*, 1989). The T7 system can be used in variety of cell types other than *E. coli*. There are reports that overproduction of some proteins is successful in a mammalian cell using the T7 system (Fuerst *et al.*, 1986; Lieber *et al.*, 1989).

적 요

자신의 promoter에 대한 특이성과 강력한 전사능력을 지닌 T7 RNA 중합효소의 존재하에서, T7 promoter의 아래에 위치해 있는 chloramphenicol acetyltransferase 유전자의 선택적 과잉발현을 이룩하였다. 또한 T7 RNA 중합효소의 copy 수를 증가시킴에 따라 T7 발현체계에서, chloramphenicol acetyltransferase의 양 역시 상응하여 증가함을 확인하였다.

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REFERENCES

1. **Davanloo, P., A.H. Rosenberg, J.J. Dunn and F.W. Studier,** 1984. Cloning and expression of the gene for bacteriophage T7 RNA polymerase. *Proc. Natl. Acad. Sci. USA*, **81,** 2035-2039.
2. **Fuerst, T.R., E.G. Niels, F.W. Studier and B. Moss,** 1986. Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. *Proc. Natl. Acad. Sci. USA*, **83,** 8122-8126.
3. **Grodberg, J. and J.J. Dunn,** 1988. *OmpT* encodes the *Escherichia coli* outer membrane protease that cleaves T7 RNA polymerase during purification. *J. Bacteriol.*, **170,** 1245-1253.
4. **Lieber, A., U. Kiessling and M. Strauss,** 1989. High level gene expression in mammalian cells by a nuclear T7-phage RNA polymerase. *Nucleic Acids Res.*, **17,** 8485-8493.
5. **McKinney, J.D., J. Lee, R.E. O'Neill and A. Goldfarb,** 1987. Overexpression and purification of a biologically active rifampicin-resistant β subunit of *Escherichia coli* RNA polymerase. *Gene*, **58,** 13-18.
6. **Nishimura, N., S. Komatsubara, T. Taniguchi and M. Kisumi,** 1987. Hyperproduction of aspartase of *Escerichia coli* K-12 by the use of a runaway plasmid vector. *J. Biotechnol.*, **6,** 31-40.
7. **Read, S.M. and D.H. Northcote,** 1981. Minimization of variation in the response to different proteins of the Coomassie blue G dye-binding assay for protein. *Anal. Biochem.*, **116,** 53-64.
8. **Remaut, E., H. Tsao and W. Fiers,** 1983. Improved plasmid vectors with a thermoinducible expression and temperature-regulated runaway replication. *Gene*, **22,** 103-113.
9. **Rosenberg, A.H., B.N. Lade, D. Chui, S. Lin, J.J. Dunn and F.W. Studier,** 1987. Vectors for selective expression of cloned DNAs by T7 RNA polymerase. *Gene*, **56,** 125-135.
10. **Rosenberg, A.H. and F.W. Studier,** 1987. T7 RNA polymerase can direct expression of influenza virus cap-binding protein (PB2) in *Escherichia coli*. *Gene*, **59,** 191-200.
11. **Shiau, A. and J.M. Smith,** 1988. Improved *cat* gene cassette for promoter analysis and genetic constructions. *Gene*, **67,** 295-299.
12. **Shaw, W.V.,** 1975. Chloramphenicol acetyltransferase from chloramphenicol-resistant bacteria. *Methods Enzymol.*, **43,** 737-755.
13. **Singer, S.C., C.A. Richards, R. Ferone, D. Benedict and P. Ray,** 1989. Cloning, purification and properties of *Candida albicans* thymidylate synthase. *J. Bacteriol.*, **171,** 1372-1378.
14. **Studier, F.W. and B.A. Moffatt,** 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.*, **189,** 113-130.
15. **Tabor, S. and C.C. Richardson,** 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. *Proc. Natl. Acad. Sci. USA*, **82,** 1074-1078.
16. **Toyoda, H., M.J.H. Nicklin, M.G. Murray, C.W. Anderson, J.J. Dunn, F.W. Studier and E. Wimmer,** 1986. A second virus-encoded proteinase involved in proteolytic processing of poliovirus polyprotein. *Cell*, **45,** 761-770.
17. **Uhlin, B.E. S. Molin, P. Gustafsson and K. Nordström,** 1979. Plasmids with temprature-dependent copy number for amplification of cloned genes and their products. *Gene*, **6,** 91-106.
18. **Williams, K.P., R. Müller, W. Rüger and E.P. Geiduschek**. 1989. Overproduced bacteriophage T4 gene 33 protein binds RNA polymerase. *J. Bacteriol.*, **171,** 3579-3582.
19. **Yasuda, S. and T. Takagi,** 1983. Overproduction of *Escherichia coli* replication proteins by the use of runaway-replication plasmids. *J. Bacteriol.*, **154,** 1153-1161.

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