ELSEVIER

Gene 190 (1997) 11–15

GENE
AN INTERNATIONAL JOURNAL ON GENES AND GENOMES

An artificial regulatory circuit for stable expression of DNA-binding proteins in a T7 expression system[1]

Bindu D. Paul, Vaidyanathan Ramesh[2], Valakunja Nagaraja *

Centre for Genetic Engineering, Indian Institute of Science, Bangalore 560 012, India

Received 4 February 1996; revised 12 June 1996; accepted 9 July 1996; Received by P. Roy

Abstract

We had earlier overproduced the transcription activator protein C of bacteriophage Mu in a phage-T7 expression system. Although we achieved a high level of overproduction, the expression was not consistent. This could be due to the leaky expression of T7 RNA polymerase in the uninduced state. Introduction of pLysS, a plasmid encoding T7 lysozyme, a natural inhibitor of T7 RNA polymerase, resulted in consistent, but extremely low production of the C protein. To overcome this problem, we have devised an artificial regulatory circuit to obtain stabilised, consistent overproduction of C protein. The C-binding site was cloned downstream from the transcription start point of T7 *lys*. Upon induction, the C protein produced binds to its site with a very high affinity, possibly acting as a transcriptional roadblock for *lys*. This would overcome the inhibitory effect of T7 lysozyme on T7 RNA polymerase.

Keywords: Bacteriophage Mu; T7 lysozyme; T7 RNA polymerase; C protein; Regulation

1. Introduction

T7-based expression systems (Studier et al., 1990) are widely used for large scale overproduction of recombinant proteins. The system makes use of the T7 RNA polymerase, which is a highly active enzyme, elongating RNA chains eight times faster than the *Escherichia coli* enzyme. (Bonner et al., 1994). Moreover, the enzyme is highly specific and selectively transcribes genes cloned under T7 promoters (Chamberlin et al., 1970). This specificity has been harnessed in transcription and expression studies both in bacterial (Tabor and Richardson, 1985; Studier and Moffatt, 1986) and eukaryotic cells (Feurst et al., 1986; Dunn et al., 1988; Wilk et al., 1992; Chen et al., 1994; Deyev et al., 1994). In spite of its many merits and wide usage, this powerful expression system poses certain problems in many cases. The most commonly encountered problems are inconsistency in levels of expression, formation of inclusion bodies and instability of clones. Inconsistency in levels of expression of target genes has been attributed to the leaky expression of T7 RNA polymerase although the gene is placed under the control of strong inducible promoters. Low levels of T7 RNA polymerase were detectable in cases where the gene for the T7 enzyme was cloned under the *lac*UV5 (Deng et al., 1990; O'Mahony et al., 1990) and λ p_L promoters (Davison et al., 1989). Basal levels of RNA polymerase present in the uninduced cell can direct sufficient expression of target genes. Thus it can prevent the establishment of foreign and especially toxic genes in the cell.

To counter the problem of leaky expression and to achieve a tight control, the Lys series of vectors were developed (Studier, 1991). These vectors harbour the gene for T7 lysozyme (Chang and Cohen, 1978). T7 lysozyme is an inhibitor of T7 RNA polymerase (Moffatt and Studier, 1987; Cheng et al., 1994). Lysozyme binds to the T7 RNA polymerase present in the uninduced cell and prevents it from transcribing

* Corresponding author. Tel. +91 80 3092598; Fax +91 80 3341683; e-mail: vraj@cge.iisc.ernet.in

[1] Presented at the International Conference on 'Eukaryotic Expression Vector Systems: Biology and Applications', National Institute of Immunology, New Delhi, India, 4–8 February 1996.

[2] Present address: Department of Biology, Massachusetts Institute of Technology, Cambridge, MA, USA.

Abbreviations: *A*, absorbance (1 cm); bp, base pair(s); C, transcription activator protein C of bacteriophage Mu; IPTG, isopropyl-β-D-thiogalactopyranoside; $lacO_2$, a *lac* operator sequence within the coding region of *lacZ*; LB, Luria-Bertani (medium); *lys*, gene encoding T7 lysozyme; PolIk, Klenow (large) fragment of *E. coli* DNA polymerase I; SDS, sodium dodecyl sulfate.

PII S0378-1119(96)00783-4

target genes, thereby preventing the leaky expression. Basal expression of T7 RNA polymerase has also been controlled using *lac* repressor to block T7 promoter (Dubendorff and Studier, 1991). More recently, the basal level of expression of T7 RNA polymerase was controlled by another approach which involved a transcription termination-antitermination mechanism (Mertens et al., 1995).

We have previously overexpressed C, a transcription activator (Hattman et al., 1985) of bacteriophage Mu in the T7 expression system. Although we obtained a high level of overproduction, the transformed clones exhibited anomalous behaviour with respect to their growth, and capability to express C (Ramesh et al., 1994a). Two kinds of colonies were observed. The fast growers, which appeared after 12 h, did not express the protein to detectable levels, whereas the slow growers, which appeared 20 h after incubation, produced the protein. However, not all slow growing colonies synthesised the protein to significant levels. The colonies which hyperexpressed C initially, lost this ability on subsequent subculturing or storage in the form of glycerol stocks and plates. To overcome this problem, the vector pLysS was introduced into the system. The clones were stable in a LysS background, but the levels of overproduction dropped down drastically, possibly due to the presence of lysozyme. Here we describe a strategy to achieve a consistent overproduction of C by bringing down the levels of lysozyme in the induced state.

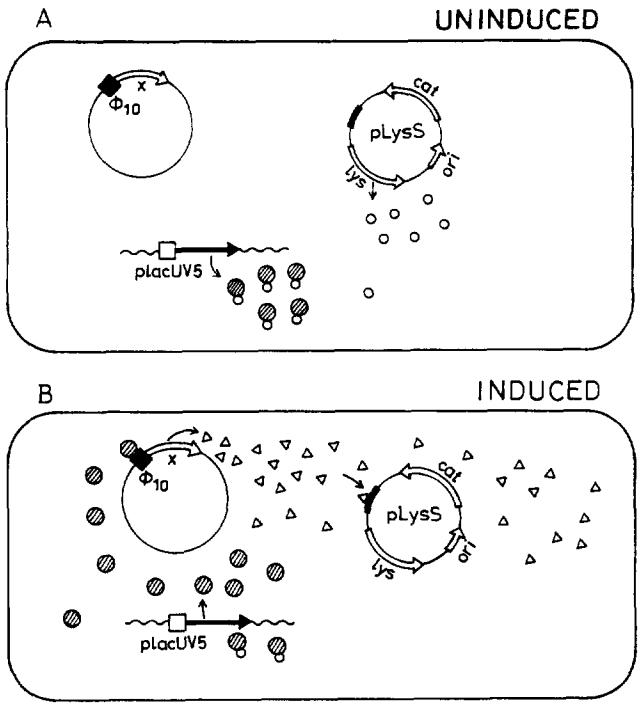

Fig. 1. Regulatory strategy. (**A**) The lysozyme produced from the vector pLysS (small open circles) binds to T7 RNA polymerase (hatched circles) in the uninduced cell and prevents it from engaging in transcription. (**B**) Upon induction, the gene for T7 RNA polymerase (heavy solid arrow) is expressed and the enzyme (hatched circles) directs transcription of gene *x* from the T7 promoter, ϕ_{10}. The protein X (open triangles) binds to its cognate site (shown as a solid box) present upstream from *lys* and shuts off the expression of *lys* gene, relieving the inhibition imposed on the polymerase. This is analogous to repressor-operator interactions.

2. Experimental and discussion

2.1. Strategy for high level expression

The regulatory circuit which would ensure downregulation of T7 lysozyme expression is depicted in Fig. 1. The approach involves the introduction of the cognate site of a site-specific DNA-binding protein downstream from transcription initiation of *lys* in the vector pLysS. The T7 lysozyme encoded by this vector binds to T7 RNA polymerase, thereby inhibiting its activity in the uninduced cell (Fig. 1A). The DNA-binding protein is not expressed, as the basal level of T7 RNA polymerase in the uninduced cell is sequestered by lysozyme. Upon induction with IPTG, T7 RNA polymerase, which is under the control of the *lac*UV5 promoter, is synthesised, and directs the transcription of the gene encoding the DNA-binding protein. The protein occupies its cognate site and hinders the transcribing *E. coli* RNA polymerase. This downregulates the expression of *lys* (Fig. 1B). As a consequence, the availability of T7 RNA polymerase for transcription of the target gene is increased.

2.2. Cloning of the protein C-binding site and expression studies

The model was tested in the case of the transcription activator protein C of phage Mu. C binds to its cognate site with a very high affinity, of the order of 2.0×10^{12} M^{-1} (Ramesh et al., 1994b). The C-binding site was cloned into the *Ban*II site of the vector pLysS as outlined in Fig. 2A. The resulting plasmid, pBN2, was used for studying the expression pattern. The protein expression profile of cells harbouring the constructs was studied after transforming the strain BL26(DE3) with the *c* gene bearing plasmid, pVR6, as described in the legend to Fig. 2B. As expected, a few fresh transformants harbouring pVR6 produced C to considerable levels (Fig. 2B, lane 1) However, on storage, the clone lost its capacity to produce the protein to detectable levels (Fig. 2B, lane 2). This loss in expression is due to the leaky expression of T7 RNA polymerase in uninduced cells as observed in other cases (Mertens et al., 1995). To control the basal levels of the polymerase in the uninduced cell and to avoid leaky expression, the vector pLysS was transformed into the cells. Use of this vector resulted in a sharp decline in C production in spite of

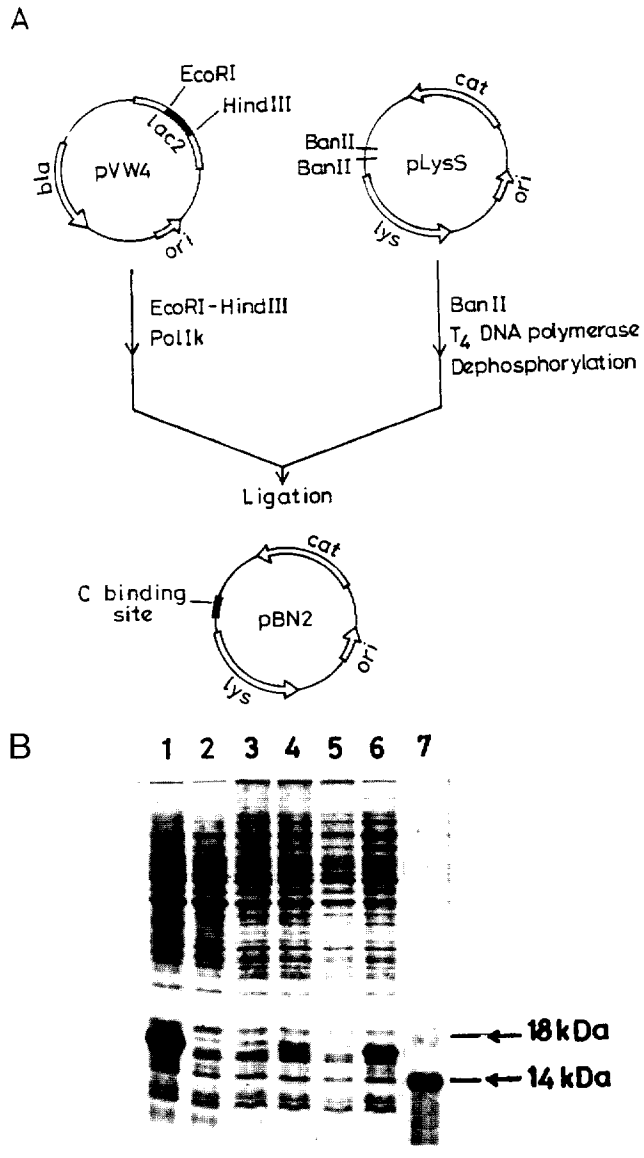

Fig. 2. Construction of plasmid pBN2 and analysis of the protein expression profile. **(A)** Construction of plasmid pBN2. **Methods:** An *Eco*RI-*Hin*dIII fragment bearing the C-binding site was released from the vector pVW4 (Ramesh et al., 1994b). The ends were filled in using PolIk. The vector pLysS was digested with *Ban*II and ends were repaired with T4 DNA polymerase. The 80-bp fragment was ligated into the blunted *Ban*II site of pLysS to generate the plasmid pBN2. **(B)** Lanes: 1, *E. coli* BL26(DE3) freshly transformed with pVR6 and induced; 2, cells induced after storage; 3 and 4, BL26(DE3) harbouring pLysS and pVR6, uninduced and induced respectively; 5 and 6, BL26(DE3) harbouring pBN2 and pVR6, uninduced and induced respectively; 7, protein molecular weight markers. **Methods:** The expression strain *E. coli* BL26(DE3) was transformed with the construct and the *c* gene bearing plasmid, pVR6. Single colonies were picked up and grown in 3 ml of LB medium to an A_{600} of 0.6. Cells were induced with 0.3 mM IPTG and grown for 2 h. The cells were harvested and lysed in SDS sample buffer (Laemmli, 1970) and equal amounts were loaded on a 15% polyacrylamide gel. Electrophoresis was performed under standard conditions (Sambrook et al., 1989).

stable maintenance of pVR6 (Fig. 2B, lane 4). When pBN2 was used, the clones produced C to significantly high levels (Fig. 2B, lane 6), although the degree of overproduction was not as high as in the case of expressing cells harbouring pVR6 alone. All the clones analysed produced C unlike the transformants obtained with pVR6 alone. The production of C was significant from 30 min onwards and peaked around 2 h (Fig. 3A). Induction beyond 2 h did not increase the levels of overproduction of C significantly (data not shown). The clones harbouring pBN2 and pVR6 continued to pro-

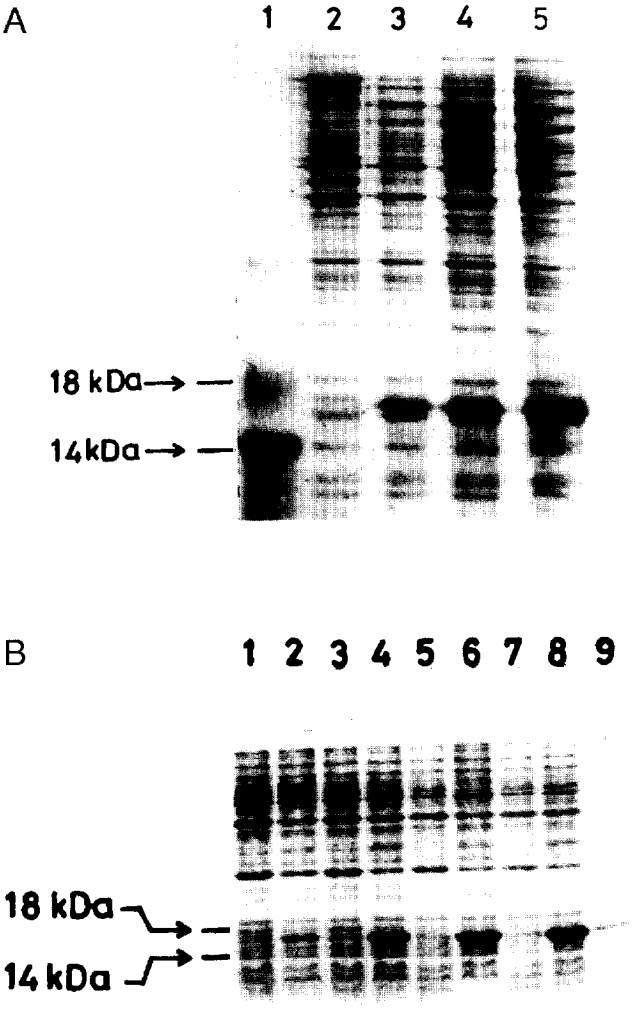

Fig. 3. Expression studies with pBN2. (**A**) Time-course of induction. *E. coli* BL26(DE3) harbouring pBN2 and pVR6 was grown to an A_{600} of 0.6 and induced with 0.3 mM IPTG at 37°C for 30 min, 60 min and 120 min (lanes 3, 4, and 5 respectively). Lanes: 1, molecular weight markers; 2, uninduced control. (**B**) Stability of expression in stored cultures. *E. coli* BL26(DE3) cells harbouring plasmids: pVR6 and pLysS (lanes 1 and 2) or pVR6 and pBN2 (lanes 3–8), stored as glycerol stocks for 6 months (lanes 1, 2, 5 and 6) and 1 year (lanes 7 and 8) or on plates (lanes 3 and 4), were analysed on a 15% polyacrylamide gel as described in the legend to Fig. 2B. Pure C protein is present in lane 9, while lanes 1, 3, 5 and 7 serve as uninduced controls.

duce C, upon induction, even after prolonged storage as glycerol stocks. Glycerol stocks revived even 1 year after storage synthesised C to high levels (Fig. 3B). This consistent, stable expression is a convenient alternative to obtain large quantities of C. Moreover, the tedious screening of fresh transformants for expressing clones is circumvented.

2.3. *Possible mechanism of* lys *repression*

The above results show that the introduction of a target sequence of a site-specific DNA-binding protein within *lys* can result in regulated expression of the cloned gene possibly due to *lys* repression. The binding of C to its site located downstream from the transcription start point of *lys* may serve as a roadblock for the transcribing *E. coli* RNA polymerase. The roadblock phenomenon has precedence in naturally occurring operons. For example, in *lac* operon, the *lacI* gene is subjected to autogenous regulation due to the presence of the operator O_I within the gene. Further, *lacZ* expression is subject to regulation at the transcriptional elongation step due to the presence of the operator $lacO_2$ within the coding region (Flashner and Gralla, 1988). Moreover, in vitro experiments with the Lac repressor have revealed its ability to block transcribing RNA polymerase and terminate transcription when its binding site has been placed downstream from the regulatory region of the *lac* operon (Dueschle et al., 1986).

To investigate this aspect, the C-binding site was introduced 90 bp away from the 3′ end of *lys* to generate pBN1 (Fig. 4A). Upon induction, it was observed that the cells harbouring pBN1 did not show a high level of expression. Presence of C-specific sequences on either side of *lys* also did not alter the production of C to appreciable levels (Fig. 4A and B). Thus, our results suggest the operation of a roadblock mechanism to bring down the levels of T7 lysozyme.

2.4. *General applicability of the system*

A vast body of literature is available on regulatory circuits and events. It is becoming increasingly clear that a regulatory protein can function either as an activator or repressor depending on the location of its specific site (Nagaraja, 1993). In this particular instance, the C protein, which functions as a transcriptional activator in its natural context, serves as a repressor for *lys* due to appropriate positioning of its specific site. We demonstrate here the utility of an artificial regulatory circuit to obtain stable and consistently high levels of expression of cloned genes, by directing a specific sequence to serve as an operator. Thus, this strategy has its potential application for the expression of any other DNA-binding protein which binds to its cognate sequence with a very high affinity. This would require the cloning of the specific site of the protein to be expressed downstream from the transcription initiation of *lys* in the plasmid pLysS. Alternately, this step can be avoided by cloning the gene encoding the DNA-binding protein downstream from *c* gene, to generate an artificial operon. It is clear that in principle any gene can be stably expressed employing the latter strategy. Efforts to achieve consistent expression of other foreign proteins is currently being investigated.

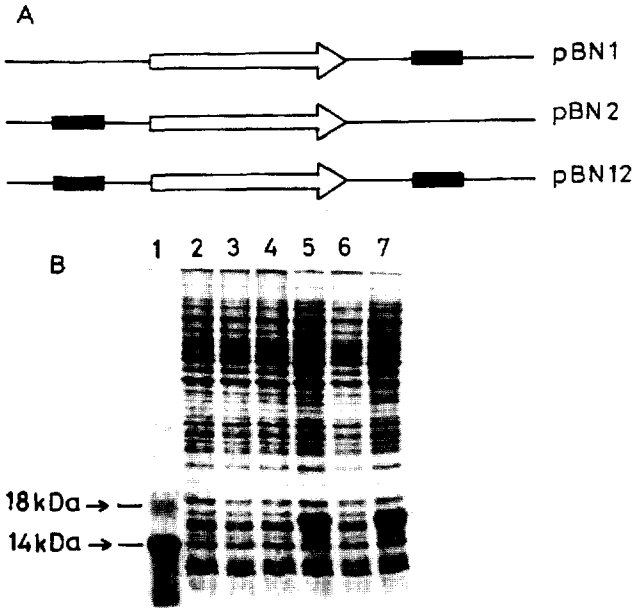

Fig. 4. Effect of the location of the C-binding site on *lys* expression. (**A**) Schematic representation of clones bearing the C-binding site at different positions relative to *lys*. The C-binding site was cloned into the *Eco*RV site of pLysS to generate the plasmid pBN1. This construct has the C-binding site 90 bp away from *lys* gene. pBN12 was constructed from pBN2 in a similar manner. This construct has the C-binding site on the 3′ end of *lys* in addition to the site present upstream from *lys*. (**B**) Expression studies with the constructs. *E. coli* BL26(DE3) cells carrying these constructs were induced, and extracts were prepared as described in the legend to Fig. 2B. Lanes: 1, protein molecular weight marker; 2, 4 and 6, uninduced controls for cells harbouring pBN1, pBN2, and pBN12, respectively; 3, 5 and 7, respective protein profiles after induction.

Acknowledgement

We thank D.R. Radha for technical assistance. B.D.P. is a senior research fellow of the Council of Scientific and Industrial Research, Government of India. V.R. is supported by the Jawarharlal Nehru Centre for Advanced Scientific Research. Grants from the Technology Development Mission, Indian Institute of Science, Department of Science and Technology, Government of India are gratefully acknowledged.

References

Bonner, G., Lafer, E.M. and Sousa, R. (1994) Characterization of a set of T7 RNA polymerase active site mutants. J. Biol. Chem. 269, 25120–25128.

Chamberlin, M., McGrath, J. and Waskell, L. (1970) New RNA polymerase from *Escherichia coli* infected with bacteriophage T7. Nature 228, 227–231.

Chang, A.C.Y. and Cohen, S.N. (1978) Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from P15A cryptic miniplasmids. J. Bacteriol. 134, 1141–1156.

Chen, X., Li, Y., Xiong, K. and Wagner, T.E. (1994) A self-initiating eukaryotic transient gene expression system based on cotransfection of bacteriophage T7 RNA polymerase and DNA vectors containing an autogene. Nucleic Acids Res. 22, 2114–2120.

Cheng, X., Zhang, X., Pflugrath, J.W. and Studier, F.W. (1994) The structure of bacteriophage T7 lysozyme, a zinc amidase and an inhibitor of T7 RNA polymerase. Proc. Natl. Acad. Sci. USA 91, 4034–4038.

Davison, J., Chevalier, N. and Brunel, F. (1989) Bacteriophage T7 RNA polymerase-controlled specific gene expression in *Pseudomonas*. Gene 83, 371–375.

Deng, T., Noel, J.P. and Tsai, M.-D. (1990) A novel expression vector for high-level synthesis and secretion of foreign proteins in *Escherichia coli*: overproduction of bovine pancreatic phospholipase A2. Gene 93, 229–234.

Deyev, S.M., Lieber, A., Radko, B.V. and Polanovsky, O.L. (1994) Expression of immunoglobulin genes tandem in eukaryotic cells under the control of bacteriophage T7 RNA polymerase. Appl. Biochem. Biotechnol. 47, 143–154.

Dubendorff, J.W. and Studier, F.W. (1991) Controlling basal expression in an inducible T7 expression system by blocking the target T7 promoter with *lac* repressor. J. Mol. Biol. 219, 45–59.

Dueschle, J., Gentz, R. and Bujard, H. (1986) *lac* repressor blocks transcribing RNA polymerase and terminates transcription. Proc. Natl. Acad. Sci. USA 83, 4134–4137.

Dunn, J.J., Krippl, B., Bernstein, K.E., Westphal, H. and Studier, F.W. (1988) Targeting T7 bacteriophage RNA polymerase to the mammalian cell nucleus. Gene 68, 259–266.

Feurst, T.R., Niles, E.G., Studier, F.W. and Moss, B. (1986) Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. Proc. Natl. Acad. Sci. USA 83, 8122–8126.

Flashner, Y. and Gralla, J.D. (1988) Dual mechanism of repression at a distance in the *lac* operon. Proc. Natl. Acad. Sci. USA 85, 8968–8972.

Hattman, S., Ives, J., Margolin, W. and Howe, M.M. (1985) Regulation and expression of the bacteriophage Mu *mom* gene: mapping of the transactivation (Dad) function to the C region. Gene 39, 71–76.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Mertens, N., Remaut, E. and Fiers, W. (1995) Tight transcriptional control mechanism ensures stable high-level expression from T7 promoter-based expression plasmids. Biotechnology 13, 175–179.

Moffatt, B.A. and Studier, F.W. (1987) T7 lysozyme inhibits transcription by T7 RNA polymerase. Cell 49, 221–227.

Nagaraja, V. (1993) Control of transcription initiation. J. Biosci. 18, 13–25.

O'Mahony, D.J., Wang, H.Y., McConnell, D.J., Jia, F., Zhou, S.W., Yin, D. and Qi, S. (1990) The effect of phage T7 lysozyme on the production biologically active porcine somatotropin in *Escherichia coli* from a gene transcribed by T7 RNA polymerase. Gene 91, 275–279.

Ramesh, V., De, A. and Nagaraja, V. (1994a) Overproduction and purification of C protein, the late gene transcription activator from phage Mu. Prot. Exp. Purif. 5, 379–384.

Ramesh, V., De, A. and Nagaraja, V. (1994b) Engineering hyperexpression of bacteriophage Mu C protein by removal of secondary structure at the translation initiation region. Protein Eng. 7, 1053–1057.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning. A Laboratory Manual, 2nd ed. CSHL Press, Cold Spring Harbor, NY.

Studier, F.W. (1991) Use of bacteriophage T7 lysozyme to improve an inducible T7 expression system. J. Mol. Biol. 219, 37–44.

Studier, F.W. and Moffatt, B.A. (1986) Use of bacteriophage T7 RNA polymerase to direct selective high level expression of cloned genes. J. Mol. Biol. 189, 113–130.

Studier, F.W., Rosenberg, A.H., Dunn, J.J. and Dubendorff, J.W. (1990) Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol. 185, 60–89.

Tabor, S. and Richardson, C.C. (1985) A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA 82, 1074–1078.

Wilk, T., Mierswa, H., Kraussich, H.G., Dunn, J.J. and Bosch, V. (1992) Expression of biologically active HIV glycoproteins using a T7 RNA polymerase-based eucaryotic vector system. Virus Genes 6, 229–246.