Journal of Microbiology and Biotechnology
Vol. 7. No. 1, 21-24, 1997

Investigation of the Relationship between Protein, Message and Inducer Concentrations in Recombinant *E. coli* Cells

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Chloramphenicol acetyl transferase (CAT) protein and mRNA levels in *E. coli* were determined following induction of a *tac::cat* construct by isopropyl-β-thiogalactopyranoside (IPTG). High *cat* mRNA levels did not directly reflect CAT protein levels, in either shakeflask experiments or fermentations. Furthermore, concentrations of IPTG resulting in the highest levels of expression of *cat* mRNA, were different to those resulting in highest levels of CAT protein. The data suggest that high transcriptional activities lead to limitations at the translational level.

Various bacterial expression systems have been designed for high-level protein production. The most efficient systems offer tight control of transcription and translation, as obtained after promoter sequence optimization (2) or optimal vector design (3). The most widely used promoters, including *tac*, pL and T7, are induced either chemically (for example by isopropyl-β-thiogalactopyranoside [IPTG]) or by a temperature increase.

The effectiveness of a bacterial expression system is determined by measuring enzyme activities or amounts of protein expressed by promoter-reporter gene fusions. However, there is ample evidence to show that efficiency of translation is not directly related to functional mRNA levels. For example, Vind *et al.* (10) showed that expression of the *lacZ* gene on a high copy number plasmid was limited at the translational level after induction with IPTG. During periods of high transcriptional activity, protein production is limited by the concentration of free ribosomes, and consequently the metabolic burden is increased. Under these circumstances, mRNA is degraded. This invariably leads to sub-optimal expression of recombinant proteins.

Elevated expression of foreign proteins in *E. coli* can result in increased activation of proteases, and therefore product degradation, due to cellular stress responses (8). Overexpression of proteins such as CAT, which is considered endogenous to some strains of *E. coli*, can lead to enhanced production of proteases identical to those induced by the stringent response (5). Thus, rapid increases in CAT concentration on derepression by 5 mM IPTG are followed by an even faster decline. Cells induced with lower IPTG concentrations did not exhibit this phenomenon. The highest level of CAT protein obtained was achieved after minimizing protease activity in a fed-batch system by simultaneous addition of glucose, phenylalanine and IPTG (8). However, neither study attempted to relate mRNA and protein levels.

Ribonucleic acid (RNA) levels are commonly quantified by filter hybridization techniques. Detection by simple experiments such as slot-blotting result in gross underestimation of specific mRNA levels, due to steric hindrance from total RNA that is loaded simultaneously onto the solid support (6). These disadvantages can be overcome by loading a series of standards onto the solid support in combination with increasing levels of commercially-purified ribosomal RNA (6).

In this work, the optimized protocol for mRNA detection has been used to quantify chloramphenicol acetyl transferase (CAT) messenger RNA expressed from a *tac::cat* transcriptional fusion in *E. coli*. CAT mRNA levels obtained at different IPTG concentrations are compared with CAT protein concentration, and the best inducer concentration for a batch system is established. Experiments are conducted in shakeflasks and simple 2-liter fermenters to determine whether the results from each system are comparable.

MATERIALS AND METHODS

Plasmid Construction

A *tac::cat* transcriptional fusion, pCT103, was con-

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Key words: CAT protein, CAT mRNA, IPTG, *E. coli*

structed by ligating a 269 bp fragment containing the *tac* promoter to the promoterless CAT reporter gene in the plasmid pPM3024 (11). Details of the construction are shown in Fig. 1. The *tac* promoter was isolated from the plasmid pKK223-3 (Pharmacia Biotech, Uppsala, Sweden). The fragment was separated on a 1.5% GTG agarose electrophoresis gel and purified using a Mermaid kit (Bresatec, Adelaide, Australia). The purified DNA was then ligated into the *Bam*HI site of the bidirectional promoter probe vector pPM3024. The resulting plasmid (pCT103) was transformed into the *E. coli* strain CB806 and plated onto ampicillin/chloramphenicol agar. Plasmid DNA from Amp^r, Cm^r colonies was isolated and the construct confirmed by sequence analysis. Plasmid pCT103 was then used to transform *E. coli* strain JM101. Plasmid pCT103 contains a ColE1 type replicon with a copy number of 20 to 30 per cell.

Bacterial Strains

E. coli JM101 (American type culture collection (ATCC no: 33876)) (F' *traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15 Δ(*pro-lac*) *supE mrcA thi* λ^-).

E. coli K-12 CB806 (F^- Δ*lacZ*$^-$ *lacY*$^+$ *galK rpsL thi recA*56 *phoA*8) (9).

Shakeflask Culture

5-liter shakeflasks containing 500 ml minimal media

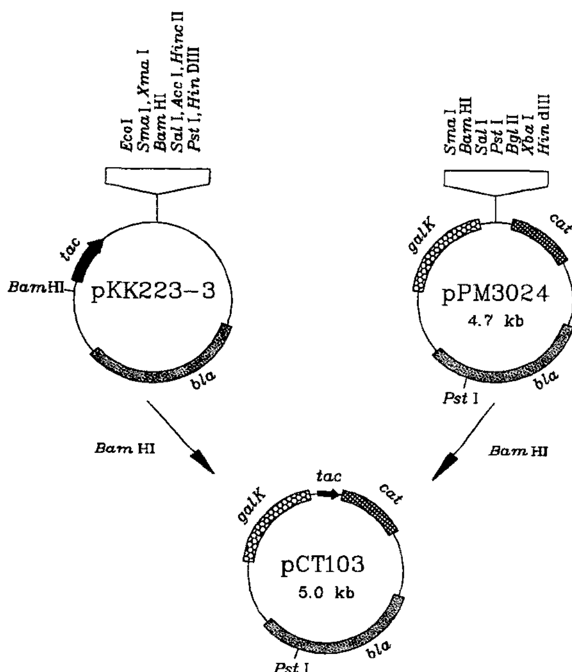

Fig. 1. Construction of a *tac::cat* transcriptional fusion.
A 269 bp fragment containing the *tac* promoter was ligated to the promoterless CAT reporter gene in the plasmid pPM3024. The *tac* promoter was isolated from plasmid pKK223-3 after *Bam*HI digestion and cloned into the *Bam*HI site of the multiple cloning site of the reporter vector pPM3024. The resulting plasmid is designated pCT103.

(NH_4Cl 2.58 g/l, KH_2PO_4 2.54 g/l, Na_2HPO_4 4.16 g/l, K_2SO_4 1.94 g/l, $FeSO_4\cdot 7H_2O$ 20 mg/l, $MnSO_4\cdot H_2O$ 5.1 mg/l, $ZnSO_4\cdot 7H_2O$ 8.6 mg/l, $CuSO_4\cdot 5\ H_2O$ 0.76 mg/l, Na_3-citrate 88.0 mg/l, HCl 0.04 ml/l, thiamine 40 μg/ml, ampicillin 100 μg/ml, glucose 3.33 g/l, $MgSO_4\cdot 7\ H_2O$ 0.67 g/l, pH 7) were inoculated 1 : 100 with an overnight culture of *E. coli* JM101, transformed with plasmid pCT103, also grown in minimal media, and cultivated at 37°C. CAT protein production was pulse induced at OD_{600nm}= 0.8 by addition of IPTG to final concentrations of 0.1, 0.4 and 1.0 mM. Samples of culture were withdrawn for analysis at 0.0, 0.5, 1.0, 2.0 and 3.0 h after induction.

Fermentations

An overnight culture of *E. coli* JM101, grown in minimal media, transformed with plasmid pCT103, was inoculated into 2-liter fermenters (Applikon, Schiedam, The Netherlands) containing 1.2 liter of minimal media (as in shakeflask experiments), to give a starting OD_{600nm} of either 5×10^{-3} or 5×10^{-5} in the fermenter. Fermenters were operated at a temperature of 37°C, pH 7, and dissolved oxygen remained above 30% of saturation at all times. CAT protein production was pulse induced at OD_{600nm}= 0.8 by addition of IPTG to final concentrations of 0.025, 0.1, 0.4 and 1.0 mM. Samples of culture were withdrawn for analysis at 0.0, 0.5, 1.0, 2.0 and 3.0 h after induction.

CAT Assay

Cells for CAT assay were collected (9800×g, 5 min), resuspended in 1 ml buffer (10 mM Tris, 1 mM EDTA, pH 7) and stored at -70°C. Frozen cells were diluted with buffer to the original volume and disrupted by two passes through a French Press at 100 MPa. Microscopic observation of cells, and analysis of homogenates by analytical disc centrifuge, confirmed the absence of inclusion bodies. Disrupted samples were treated with detergent (Triton X-100, supplied with the CAT-ELISA kit) at room temperature for 30 min. Previous work has demonstrated that this method maximizes CAT protein recovery (7). Insoluble debris was sedimented (13000×g, 10 min) and CAT protein in the supernatant was measured using a CAT-ELISA kit (Boehringer Mannheim GmbH, Mannheim, Germany). This kit uses a polyclonal antibody to CAT, that will detect intact CAT protein and degraded fragments that contain an intact epitope.

Protein Assay

Total soluble protein was measured using the BCA kit from Pierce (Rockford, USA).

RNA Isolation and Detection

RNA was extracted from cells as previously described (6). Cells were collected (9800×g, 5 min) and stored as a pellet at -70°C. Slot-blotting, hybridization, RNA detection by chemiluminescence and CAT mRNA quantitation were done as previously described. CAT mRNA standards and the DIG-labelled complimentary RNA probe were constructed as described previously (6).

RESULTS AND DISCUSSION

CAT protein levels and corresponding mRNA levels from shakeflask experiments are shown in Figs. 2 and 3. Protein production was initiated at OD_{600nm}=0.8 by adding IPTG as described in Materials and Methods. CAT protein production was monitored for three hours. CAT mRNA and protein were detected almost immediately after induction with IPTG. The highest levels of CAT mRNA were obtained using IPTG inducer at a concentration of 1 mM. However, CAT mRNA levels decreased significantly 1 h after induction for inducer concentrations above 0.1 mM. By comparison, all inducer concentrations used resulted in steady accumulation of CAT protein over the 3 h of the experiment, although the rate of accumulation was slower at the higher inducer concentrations. The highest levels of CAT protein accumulation were detected at 0.1 mM IPTG.

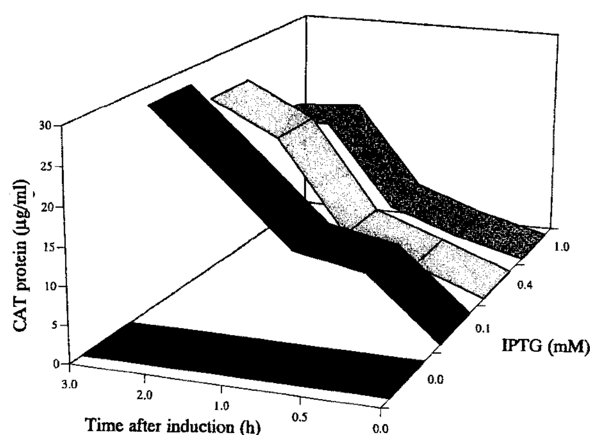

Fig. 2. CAT protein concentrations expressed as μg/ml of culture versus time after induction of gene expression by four different levels of IPTG in shakeflask experiments.

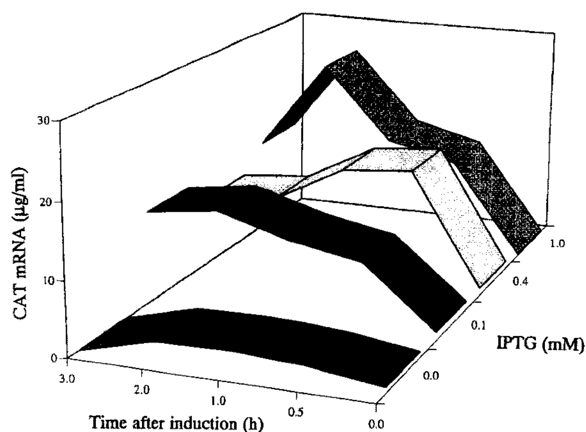

Fig. 3. CAT mRNA expressed as μg/ml of culture versus time after induction of gene expression by four different levels of IPTG in shakeflask experiments.

The results obtained with shakeflask experiments were repeated using 2-liter fermenters in order to determine whether the two systems were comparable. The results for CAT mRNA and corresponding protein levels are shown in Figs. 4 and 5. As was the case for shakeflask cultures, expression of mRNA and protein was initiated immediately after induction of *cat* with IPTG. The patterns of expression of CAT mRNA and protein were also similar to those obtained for shakeflask cultures. No significant increases in cell mass were observed following induction for either the shakeflask or fermenter culture systems.

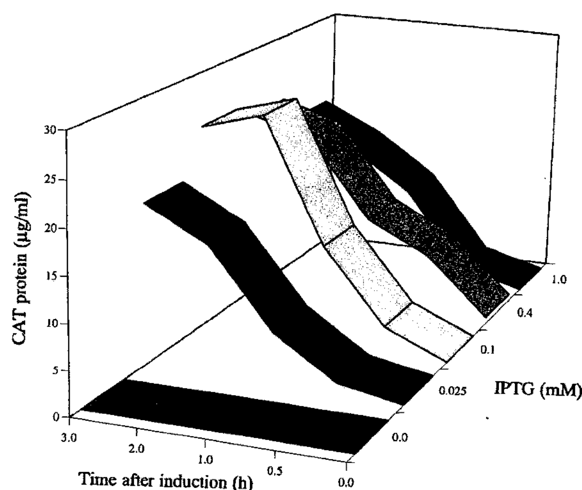

Fig. 4. CAT protein concentrations expressed as μg/ml of culture versus time after induction of gene expression by five different levels of IPTG in fermentation experiments.

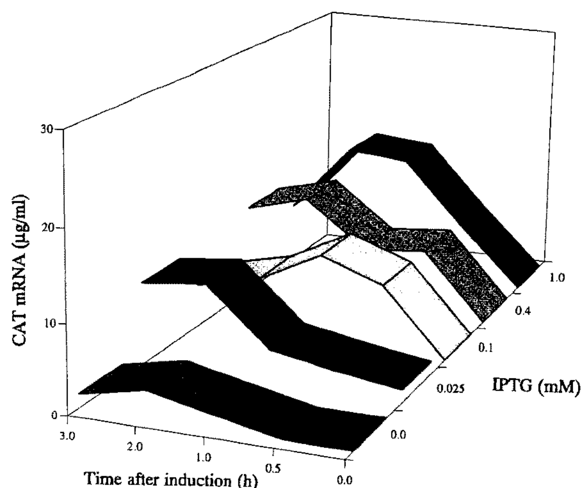

Fig. 5. CAT mRNA versus time expressed as μg/ml of culture after induction of gene expression by five different levels of IPTG in fermentation experiments.

The fact that the highest levels of CAT protein accumulation were achieved at low levels of IPTG inducer (0.1 mM), and that an increase in IPTG concentration resulted in a concomitant increase in mRNA level but not CAT protein, suggests that CAT protein production is severely limited at the translational level. This is to be expected, since during periods of high transcriptional activity brought about by induction of expression of proteins from a strong promoter, such as *tac*, protein production will be limited at the translational level because of competition for free ribosomes. A similar phenomenon has been shown to occur during expression of LacZ from the *lac* promoter by Vind *et al.*, (10). These results may also be explained in part by activation of endogenous proteases. In *E. coli*, proteases such as La are induced by stress (4). Consequently, when IPTG is used to induce a state of high transcriptional activity, expression of proteases leads to reduced levels of CAT protein (8). In the present work, this will only be a concern if epitopic sites to the polyclonal antibody on the CAT protein are degraded (see Materials and Methods).

Determination of an optimal inducer concentration is a complex process. Previous studies have shown that CAT protein production is dependent on the time and mode of induction (1, 8). It would be expected that this response is not only growth related but is also dependent on recombinant plasmid copy number and the concentration of cells, and thus oxygen availability, at induction. Further work is being conducted to investigate the effect of oxygen availability on expression of CAT under different induction conditions.

Our work illustrates the mRNA "log jam" effect produced when genes are expressed from multicopy recombinant plasmids. The ribosomal machinery cannot effectively meet the metabolic and energy requirements necessary to effectively process mRNAs for maintenance and recombinant protein needs. Given that we use a relative low copy number plasmid to express CAT, this situation would be expected to be worse for the common high copy number expression vectors currently available.

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(Received August 16, 1996)