Induction of T7 Promoter at Higher Temperatures May Be Counterproductive

Priyanka Namdev¹ · Hamid Y. Dar^{2,3} · Rupesh K. Srivastava^{2,3} ·
Rajesh Mondal^{4,5} · Rajaneesh Anupam¹

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Abstract Bacterial expression of recombinant proteins is the most popular and convenient method for obtaining large quantities of pure protein. The induction of T7 promoter with isopropyl- β -D-thiogalactopyranoside (IPTG) is widely used for expression of large quantities of proteins in *Escherichia coli*. It has been reported that basic T7 promoter is leaky and expresses cloned genes without induction. The effect of T7 promoter induction on expression of proteins at different temperature using flow cytometry has not yet been investigated. Green fluorescent protein (GFP) as a non-peptide tag can be used for protein solubility screening and for high-throughput optimization of expression conditions using flow cytometry. Therefore, flow cytometry was used to study the effect of induction on the expression of T7 promoter driven emerald GFP (emGFP) at various temperatures. We noticed that percentage of emGFP positive cells decreased instead of increasing upon induction at higher temperatures. Western blot analysis confirmed that the amount of total and soluble emGFP decreased in induced cells compared uninduced cells at

higher temperatures. Our results indicate that induction of basic T7 promoter at higher temperature may not necessarily increase protein expression. While using a basic T7 promoter it is highly recommended to analyze the effect of induction on protein expression at various temperatures.

Keywords Expression · Flow cytometry · Induction · T7 promoter · emGFP

Introduction

Expression of recombinant proteins in bacterial expression system, especially *Escherichia coli* (*E. coli*), has enabled structure–function relationship studies of numerous proteins and production of large quantities of therapeutically important proteins. Many varieties of *E. coli* strains and plasmid vectors with different promoters for induction of expression and different tags for detection and purification are available that are tailored to meet specific requirements

✉ Rajaneesh Anupam
rajaneeshanupam@gmail.com

Priyanka Namdev
priyankanamdev01@gmail.com

Hamid Y. Dar
hamidzoo87@gmail.com

Rupesh K. Srivastava
rupesh_srivastava13@yahoo.co.in

Rajesh Mondal
drrajeshmondal@gmail.com

² Department of Zoology, School of Biological Sciences, Dr. Harisingh Gour Central University, Sagar, M. P. 470003, India

³ Present Address: Department of Biotechnology, All India Institute of Medical Sciences, New Delhi 110029, India

⁴ Department of Microbiology, School of Biological Sciences, Dr. Harisingh Gour Central University, Sagar, M. P. 470003, India

⁵ Present Address: Bacteriology Division, National Institute for Research in Tuberculosis, Chennai, T. N. 600031, India

¹ Department of Biotechnology, School of Biological Sciences, Dr. Harisingh Gour Central University, Sagar, M. P. 470003, India

of laboratories [1]. The basic T7 promoter is one of the most popular promoters used in many laboratories for expression of recombinant proteins in bacteria. However, it is documented that widely used T7 promoter can be leaky and express protein without induction by isopropyl- β -D-thiogalactopyranoside (IPTG) with inconsistent levels of expression upon induction [2]. The effect of induction at various temperatures on expression of protein under the influence of the T7 promoter has not been fully investigated.

Green fluorescent protein (GFP) provides the ability to study the impact of temperature with respect to T7 promoter induction on protein expression using flow cytometry and various other high throughput screening assays. In addition, engineered GFP is one of the many non-peptide tags which could be used as a solubility enhancer when fused to the insoluble protein of interest [3–5]. The fluorescence of GFP is dependent on its proper folding which is affected when fused to the insoluble proteins [6, 7]. Therefore, GFP is fused to the C-terminus of expressed proteins not only to enhance the solubility, but also as a marker of solubility. However, wild type GFP tends to go into insoluble fraction when expressed at 37 °C, limiting its use. Therefore, emerald GFP (emGFP), developed by Invitrogen™, has better fluorescence and solubility at 37 °C [8] and would be an ideal model protein to study the effect of temperature with respect to T7 induction on its expression.

Materials and Methods

To understand if temperature affects the expression of proteins under the influence of T7 upon induction, flow cytometry analysis was carried out to estimate the expression of emGFP from a T7 promoter based bacterial expression plasmid, pRSET/EmGFP by Invitrogen™ with and without induction at various temperatures. The N-terminal detection tag, Xpress™ epitope, was used for Western blotting. BL21 Star™ (DE3) *E. coli* cells (Invitrogen, USA) were transformed with pRSET/EmGFP plasmid using standard heat shock transformation protocol. An overnight culture of transformed cells was diluted to obtain an OD₆₀₀ of 0.07–0.1 and incubated at 37 °C until OD₆₀₀ ~ 0.6 to 0.7 was attained. To analyze the effect of IPTG induction at various temperatures on emGFP expression using fluorescence, the cultures were either induced with 0.5 mM IPTG (Sigma Aldrich, USA) or left uninduced and incubated at 15, 25 or 37 °C for 6 h. Fluorescence was used as an indicator of the amount of protein expression, folding and solubility of emGFP.

Flow cytometric analysis was performed to measure the number of fluorescent cells by exciting samples with a

488 nm laser and fluorescence was detected at 530 ± 30 nm using flow cytometer (BD FACS Aria III, BD Biosciences USA). Flow cytometric data were analyzed by using Flowjo™ 10 software (Flowjo, LLC USA). To estimate the total emGFP expression, a pellet of one milliliter bacterial culture was resuspended and boiled in standard 1X SDS-polyacrylamide gel electrophoresis loading buffer. The soluble fraction was obtained by sonicating cells in lysis buffer (1X PBS, 20% glycerol, 1% Triton X-100 and 1 mM PMSF). The whole cell lysate and soluble fractions were separated by 12% SDS-PAGE and transferred on PVDF membrane. The membranes were probed with anti-Xpress™ epitope antibodies (1:5000 dilution, Invitrogen USA) followed by HRP tagged secondary antibodies (1:20,000 dilution, Life Technologies CA).

Results and Discussion

The histograms shown in Fig. 1a is representative data of six independent experiments showing the effect of induction on the percentage of fluorescent cells with respect to temperature and the histogram of untransformed cells indicates that fluorescence observed is due to expression of emGFP and not due to auto fluorescence of bacteria. Dot plots corresponding to the histogram are shown in Fig. 1b. The average percentage of fluorescent cells (n = 6) upon induction with respect to uninduced cells at various temperatures is shown in Fig. 1c. It was observed that the percentage of fluorescent cells was almost the same in uninduced and induced samples at 15 °C. Interestingly, at 25 °C and upon induction, the average percentage of emGFP positive cells decreased by 52.24% compared to uninduced cells. Further, at 37 °C and upon induction, the average percentage of fluorescent cells was significantly decreased by 62.92% in comparison to uninduced cells. The flow cytometric data are suggesting that induction of T7 promoter at higher temperature is decreasing the amount of emGFP expression. The reduction in GFP positive cells could be due to reduction of emGFP expression upon induction at higher temperature or due to misfolding or aggregation of expressed emGFP proteins. Western blot of total cell lysate and soluble fraction was performed to determine whether the reduction in fluorescent cells is due to reduced emGFP expression or misfolding or aggregation of emGFP upon induction at higher temperature. Western blots (Fig. 1d) comparing the total amount of emGFP from whole cell lysate (top panel) in uninduced and induced cells at various temperatures clearly indicates that upon induction, expression of emGFP is decreasing instead of increasing. This difference was more pronounced at 25 and 37 °C, which corroborates with the flow cytometry data. Since fluorescence of cells is due to properly folded and

Fig. 1 Flow cytometry and Western blot analysis showing the effect of T7 promoter induction on protein expression at various temperatures. **a** Representative histograms of flow cytometry experiments ($n = 6$) showing the percentage of emGFP positive cell of samples that are either uninduced or induced with IPTG at difference temperatures as indicated. The histogram of untransformed cells is shown as control. The flow cytometry data was collected by exciting the cells at 488 nm and measuring fluorescence at measured at 530 ± 10 nm of 10,000 events. The histogram clearly shows that percentage of emGFP positive cells have significantly reduced when induced at higher temperatures. **b** Dot plots corresponding to the histogram are shown. **c** The bar graph is the average data of flow cytometry experiments ($n = 6$) comparing the percentage of

emGFP positive cells of samples that were uninduced (UI) with induced (IN) samples at various temperatures as mentioned in the graph. To compare the reduction of emGFP positive cells in induced samples graph was plotted by normalizing the percentage of fluorescence of induced samples to percentage of emGFP positive cells of uninduced samples. **d** The western blot analysis to assess the effect of induction on protein expression at various temperatures was performed using Xpress epitope. The upper panel is the western blot of weight normalized whole cell lysate of samples with and without induction at various temperatures as mentioned in the figure. The lower panel is the western blot of soluble fraction showing the amount of protein present in samples as mentioned in the figure

soluble emGFP protein, we wanted to investigate if there was any difference in soluble emGFP between uninduced and induced samples at various temperatures. The Western blot data of soluble fraction of the cells before and after induction at various temperatures is shown in Fig. 1d, bottom panel. Densitometry analysis (using AlphaEase FC 2200 software) of the bands indicate that the amount of soluble GFP does not change significantly upon induction at 15 °C, whereas at 25 and 37 °C, the amount of soluble emGFP reduced to approximately by 21% and 63%, respectively in agreement with flow cytometry data.

All the data corroborates that T7 promoter is a leaky promoter and the leakiness of the promoter increases with the increase in temperature as the amount of total emGFP in uninduced cells increased with increase in temperature (Fig. 1c). As observed from the flow cytometry and Western blotting data, induction at 15 °C has very minimal

effect on protein expression. The possible reason would be that at lower temperature the metabolism of the cell is so slow that induction had no effect on protein production rate. However, induction at 25 and 37 °C, which is supposed to increase the protein expression, instead decreased the expression of protein. Altogether, our data indicates that leakiness of the T7 promoter results in expression of large amounts of protein without induction. Moreover, the expressed protein under the influence of T7 promoter is emGFP, which is engineered to have better stability and solubility. Therefore, the data generates the necessity to revisit the effectiveness of IPTG induction of T7 promoter especially at higher temperatures. The genetic integrity of BL21 strains used for expression could influence the expression patterns. Sample to sample variation was also noticed in terms of percentage of fluorescent cells and different populations of varying fluorescent intensity (two

peaks observed in uninduced samples at 25 and 37 °C in Fig. 1a). This indicates that folding of the expressed protein differs from clone to clone. In addition, the populations of cells with different fluorescence intensity were changing upon induction and with temperature, indicating that induction and temperature play an important role in proper folding of the expressed protein. It is generally accepted that T7 promoter-based induction and expression of insoluble protein should be done at lower temperatures [9]. Here, we report that a protein engineered for better solubility shows reduced expression at higher temperatures upon induction especially under leaky promoter. Based on these observations using emGFP protein and flow analysis, it is highly recommended to optimize the time, IPTG concentration and temperature conditions for expression of recombinant proteins (especially insoluble proteins) by fusing it to fluorescent proteins. This will enable the utilization of the high throughput flow cytometry technique to optimize the expression conditions, which can be very time consuming if done using conventional SDS-PAGE analysis followed by Coomassie staining or Western blotting [10].

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Compliance with Ethical Standards

Conflict of interest All the author have contributed to the manuscript and consented for the submission and declare no conflict of interest.

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