BMB
reports

Quantification of the galactose-operon mRNAs 5 bases different in their 5'-ends

Sang Chun Ji, Xun Wang, Heung Jin Jeon, Sang Hoon Yun, Hee Jung Lee[#] & Heon M. Lim[*]

Department of Biological Science, College of Biological Sciences and Biotechnology, Chungnam National University, Daejeon 305-764, Korea

Three assay methods for quantification of the two galactose-operon mRNAs that only differ by 5 bases in their 5'-end are presented. The 5' ends of each mRNA were extended by ligating the 3'-end of the abundant 5S rRNA. This ligation extends the 5' ends of the two *gal* mRNAs long enough to be distinguished by the specific PCR primers in the following quantification reactions. Quantification of the corresponding cDNAs was performed either by primer extension assay or real-time qPCR. To circumvent the problem of the RNA ligation reaction (i.e. very low ligation efficiency), we devised a new method that employs real-time qPCR directly for the quantification of the *gal* transcripts which differ by 5 bases in their 5'-ends. [BMB reports 2010; 43(7): 474-479]

INTRODUCTION

The galactose operon of *Escherichia coli* has two promoters separated by 5 base pairs, *P1* and *P2* (Fig. 1) (1, 2). These two promoters drive transcription of the 4 structural genes, *galE, T, K*, and *M*, of the operon. The cAMP receptor protein (CRP) activates the promoter *P1*. The repressor of the operon, GalR, binds to the two operator sites separated by 113 base pairs, and brings the two operator sites close, forming a nucleoprotein structure named repressosome in which the intervening DNA sequence, including the two promoters, is looped (3). Formation of the repressosome that requires HU protein bound at the central region of the intervening sequence (4) inactivates transcription from the two promoters simultaneously (5, 6). Thus, the regulation over the two promoters has been well established (7). However, in order to understand the regulation over the two promoters being executed inside of a growing cell, one might need information over the changes in the amount of the *gal* transcripts from the two promoters.

*Corresponding author. Tel: 82-42-821-6276; Fax: 82-42-822-9690; E-mail: hmlim@cnu.ac.kr
[#]Current address; Diagnostics manufacturing team, Green Cross Medical Science Corp., Eumseong, Korea (S)

Received 5 May 2010, Accepted 4 June 2010

Keywords: Galactose operon, Real-time qPCR, Transcription, 5' RACE

Quantitative measurement, such as real-time qPCR (8), of the *gal* transcripts from the two promoters is not an easy task because the only difference between the *P1*- and *P2*-initiated transcripts is the 5 bases in their 5' ends: the *P2*-transcript has 5 more bases than the *P1*-transcript. One of the well-established assay methods on the 5' ends of RNA transcript is Randomly Amplified cDNA Ends (RACE) (9). It employs an RNA ligation reaction, in which the 3'-end of an RNA aptamer is ligated to the 5'-ends of the mRNAs in the sample. The aptamer-ligated mRNAs are copied into cDNA using a reverse-transcription reaction. The target cDNA is amplified by PCR and visualized by agarose gel electrophoresis. This method was, however, unsuitable to distinguish the two transcripts (due to 5 base difference), and turned out not to be quantitative enough for *in vivo* measurement of *gal* transcripts (because of the agarose gel electrophoresis). In this study, we developed new RACE assays suitable for quantitative measurements of the two *gal* transcripts. We also present in this report a new way of quantitative measurement on the two transcripts using real-time qPCR.

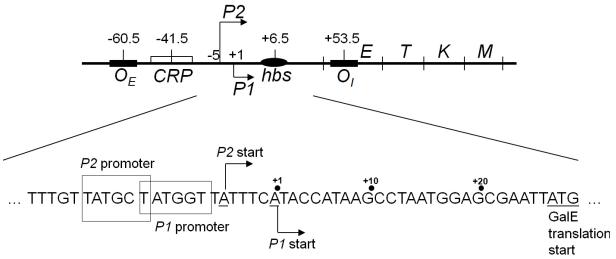


Fig. 1. A schematic presentation of the galactose operon. The -10 regions of the *P1* and *P2* promoters are shown in boxes. The transcription initiation sites are indicated by arrows and underlined. CRP points to its DNA binding site. O_E and O_I designate the operators where GalR binds. HU protein binds to *hbs*. The transcription initiation site of the *P1* promoter is assigned as +1, Nucleotide positions of the operon, thus, have been assigned relative to the *P1* initiation site.

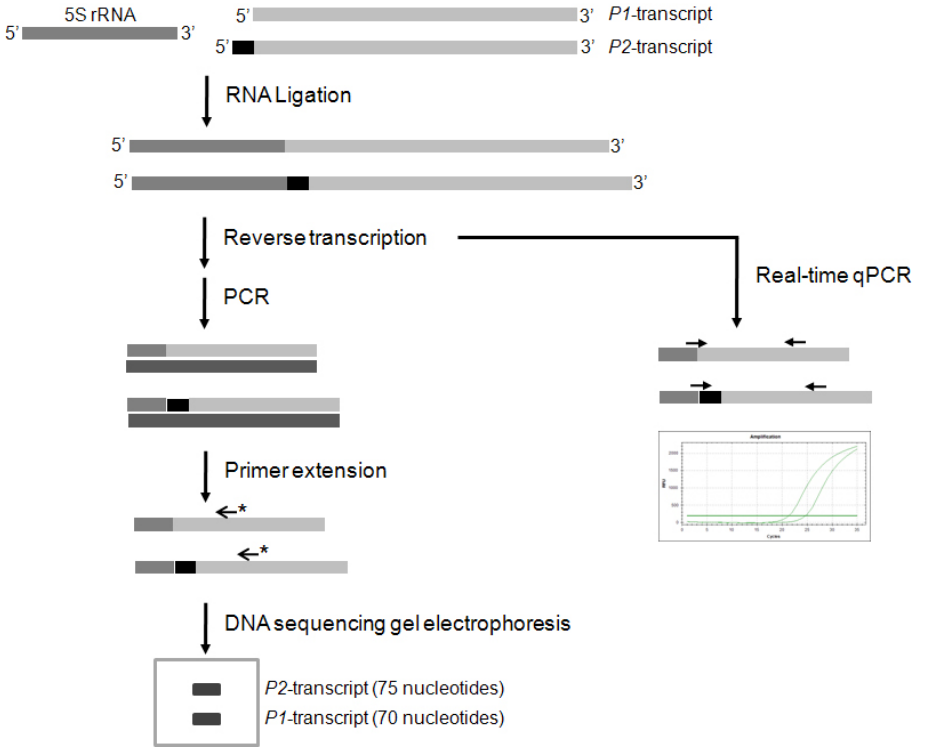


Fig. 2. A schematic presentation of the experimental steps in the RACE primer extension assay and the RACE real-time qPCR assay. Details are in the text. Both assays have a common procedure of RNA ligation and reverse transcription. After the reverse transcription, the RACE primer extension assay follows the left column procedures, but the RACE real-time qPCR assay directly goes to the real-time qPCR shown on right column. The horizontal small arrows indicate PCR primers. The arrows with *indicate the primers for the primer extension reaction.

RESULTS

RACE primer extension assay

Throughout this study, the total RNA was isolated from 2 × 10^8 cells of *E. coli* grown in LB containing 0.5% galactose that fully induces *gal* operon expression. In a conventional RACE assay, a synthetic RNA aptamer of 20-mer is ligated to the 5′-end of the total RNA. But we simply performed a ligation reaction on the total RNA. Our intension was to ligate the 3′-end of the abundant 5S rRNA in the total RNA preparation to the 5′-end of the mRNA (Fig. 2). This ligation reaction was followed by a reverse transcription using a random hexamer as a primer for cDNA synthesis. The *gal*-specific cDNA was amplified using a synthetic primer complementary to the 3′ end of the 5S rRNA and a *galE*-specific primer. Finally, visualization and quantification of the *gal* transcripts were performed by the primer extension reaction, followed by DNA-sequencing gel electrophoresis. Since the 3′ end of the primer in the primer extension reaction corresponds to +30 residue of *galE* (+1 as the *P1* transcription initiation site), and its 5′ end was radioactively labeled by ^{32}P, the mRNA from the *P1* and *P2* promoters showed up as 70 and 75 nucleotide-long DNA bands, respectively, in a film exposed to the sequencing gel (Fig. 3A). In the WT strains (MG1655, and MG1655*kan*), under the growth conditions described above, transcription from the *P1* promoter was 76.4% (+/- 5.6) and that from *P2* was 23.4% (+/- 5.6) of the total *gal* transcription (Fig. 3A). In a mutant strain, MG1655*kanP1*$^+$*P2*$^-$, in which the *P2* promoter was inactivated by a single nucleotide change (10, 11), almost 100% of the transcription was initiated from *P1*, as expected (Fig. 3A). In another mutant strain, MG1655*kanP1*$^-$*P2*$^+$, in which the *P1* promoter has been inactivated by a single nucleotide change (10, 11), most of the *gal* transcription was initiated

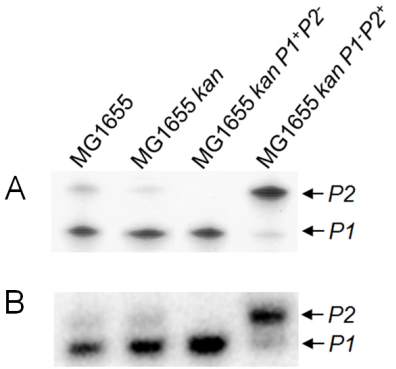


Fig. 3. Results of the RACE primer extension assay (A) and the RNase protection assay (B). DNA bands quantitatively representing the *P1*-and *P2* transcripts are indicated by arrows. The bacterial strains used to prepare total RNA are shown above each lane.

from *P2* (Fig. 3A).

Next, we tried to validate the result (as shown in Fig. 3A) from the RACE primer extension assay with the RNase protection assay (12) (See materials and methods) that does not require RNA ligation or the PCR reaction, which could inadvertently add complexity to the experimental procedures. Results, as shown in Fig. 3B, demonstrated basically the same ratio of *P1* and *P2* transcripts as shown in Fig. 3A, suggesting that the RACE primer extension assay could be used to measure the ratio of transcription from the two promoters.

RACE real-time qPCR assay

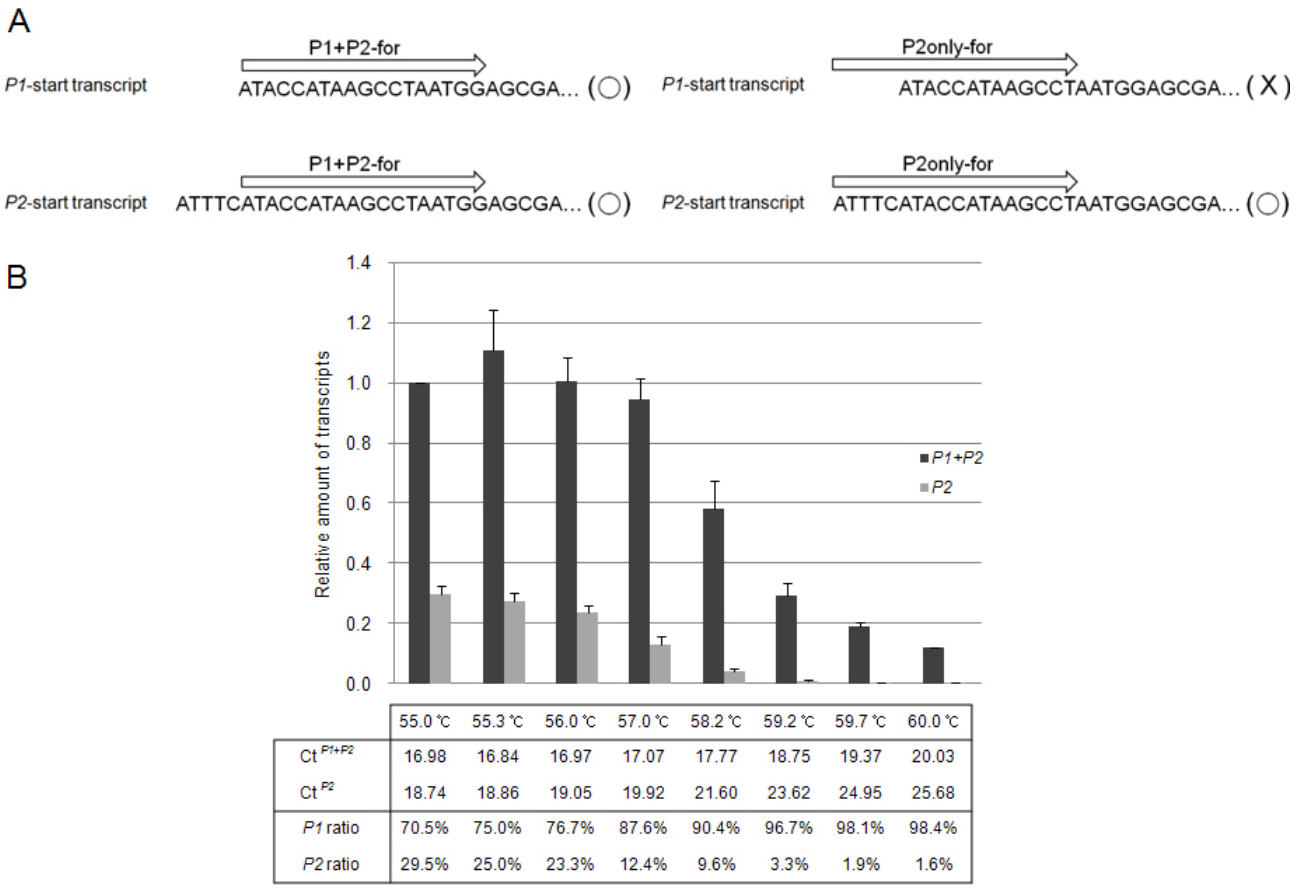
We reasoned that replacing the PCR amplification of the cDNA and the primer extension reactions in the RACE primer extension assay elaborated above with real-time qPCR would create a better quantitative assay in measuring *gal* transcripts. Thus, the total RNA ligation was performed and cDNA was prepared as described for the 5′ RACE assay above followed by real-time qPCR (Fig. 2). We were able to distinguish *P1*- and *P2*-initiated transcripts in real-time qPCR for the following reasons and via the following methods. The first 5 ribonucleotide residues of the *P1*-initiated transcript are 5′-AUACC and those of the *P2*-initiated transcript are 5′-AUUUC (The different bases are underlined). Thus, we made a forward primer (P1-for, 5′-GAGTAGGGAACTGCCAGGCATATAC-3′) of 25 nucleotides. The 21 nucleotides from the 5′-end of this primer is complementary to the 3′-end of the 5S rRNA, and the rest sequences are ATAC-3′. Thus, this primer can bind to the 5′ end of the *P1*-initiated transcripts (now ligated to the 5S rRNA), and can provide a free 3′-OH group for the following DNA polymerization reaction in real-time qPCR. Another forward primer (P2-for, 5′-GAGTAGGGAACTGCCAGGCATATTT-3′) for the *P2*-initiated transcript has the ATTT-3′ sequence at the 3′-end. The average threshold cycle (Ct) of 4 independent experiments for the *P1*-transcript was 25.13 (+/- 0.72) and that of the *P2* transcript was 26.64 (+/- 0.65). Thus, the average threshold difference was 1.51 (+/- 0.47), suggesting that there were 2.85 (+/- 1.38) times more *P1*-transcript inside of *E. coli* cells growing exponentially. Based on the threshold difference, we calculated the relative amounts of the *P1*- and *P2*- transcripts. When the relative amount of the *P1*-transcript was assumed to be 1.0, that of *P2*-transcript could be calculated by $2^{-\Delta Ct}$. Since the average threshold difference (ΔCt) was 1.51, the relative amount of the *P2*-transcript was 0.35. These calculations suggested that the *P1*- and *P2*-transcripts take 73.6% (+/- 6.3) and 26.4% (+/- 6.3) of the total *gal* transcripts, respectively. These measurements were in good agreement with the results from the RACE primer extension and RNase protection assays described above, suggesting that the RACE real-time qPCR could be used to quantify the *gal* transcripts.

Real-time qPCR only

Both methods described above start with a RACE assay that requires the RNA ligation reaction that might lower the number of cDNA templates (*gal* transcripts) for the following DNA amplification procedures (See Discussion). Thus, we devised a new set of PCR primers that would be able to distinguish the *P2*-transcript from the *P1*-transcript during the real-time qPCR procedure to eliminate the RNA ligation reaction.

First, we designed a forward primer named 'P2only-for' that is an 18-mer with a sequence (from −5 to +13 of the *gal* operon, Fig. 4A) complementary to the 3′-end of the P2-cDNA (5′-end of the *P2*-transcript). Another forward primer named 'P1&P2-for' is an 18-mer with a sequence (from +1 to +18 of the *gal* operon, Fig. 4A) complementary to the 3′-end of both the P1-and P2-cDNA. We reasoned that at an appropriate annealing temperature, the P2only-for primer would preferentially hybridize to the P2-cDNA, yielding quantitative information of the *P2*-transcript during real-time qPCR (Fig. 4A). Since the P1&P2-for primer would be able to hybridize both P1- and P2-cDNA, we expected that it should yield quantitative information of the total *gal* transcripts. Once we obtained the information, the amount of the *P1*-transcript was expected to be calculated by subtracting that of *P2*-transcript from the total transcript.

In order to determine the optimum annealing temperature for the P2only-for primer, two reactions of real-time qPCR that employ cDNA prepared from total RNA as the DNA template were set up: one with the P1&P2-for primer and the other with the P2only-for primer. The real-time qPCR reactions were subjected to various annealing temperatures from 55°C to 60°C in 8 steps. Fig. 4B shows the result of quantification. The total *gal* transcript maintained more or less the same amount in temperatures from 55°C to 56°C, and started to decrease significantly as the annealing temp reached 58°C, at which the total *gal* transcript became half that of at 55.3°C. The amount of the total transcript continued to decrease as the temperature increased. Thus, we considered the Ct values from temperatures of 55.0, 55.3, and 56°C. The average Ct of the total *gal* transcript at these temperatures was 16.93 (+/- 0.08), and that from the 'P2only-for Real-time qPCR' was 18.88 (+/- 0.16). Thus, the average ΔCt was 1.95 (+/- 0.17). When the relative amount of the total transcript was assumed to be 1.0, that from the 'P2only-for Real-time qPCR' was 0.259 (+/- 0.03). These calculations suggested that the relative amount between *P1*- and *P2*-transcript was 74.1% (+/- 3.0) and 25.9% (+/- 3.0), respectively. Considering the relative amount of the values of the *P1*- and *P2*-transcript: 76.4% (-/+ 5.6) and 23.4% (+/- 5.6) by the RACE primer extension assay, 73.6% (+/- 6.3) and 26.4% (+/- 6.3) by the RACE real-time qPCR assay, the relative amount values of the *P1*- and *P2*-transcript measured by the real-time qPCR only is in good agreement with the other two quantification methods described above. Thus, the optimum annealing temperature for 'P2only-for' primer should be in between 55°C and 56°C (Fig. 4B).



	55.0 ℃	55.3 ℃	56.0 ℃	57.0 ℃	58.2 ℃	59.2 ℃	59.7 ℃	60.0 ℃
Ct^{P1+P2}	16.98	16.84	16.97	17.07	17.77	18.75	19.37	20.03
Ct^{P2}	18.74	18.86	19.05	19.92	21.60	23.62	24.95	25.68
P1 ratio	70.5%	75.0%	76.7%	87.6%	90.4%	96.7%	98.1%	98.4%
P2 ratio	29.5%	25.0%	23.3%	12.4%	9.6%	3.3%	1.9%	1.6%

Fig. 4. The primer positions for the real-time qPCR with P2only-for primer (A) and the effect of different annealing temperatures in the real-time qPCR (B). The real-time qPCR primers' hybridization locations over the 5′ ends of the *P1*- and *P2* transcripts are indicated by arrows (A). If the hybridization is expected to be stable enough to lead to PCR amplification, it is noted with (O), if not it is noted with (X). The relative quantities of the total *gal* transcript (dark bar) and those of the *P2*-transcript (gray bar) were presented above the annealing temperature used in the real-time qPCR with the P2only-for primer. The resulting threshold cycle (Ct) and transcription ratio between *P1* and *P2* promoters calculated upon the Ct values is presented in a table under the bar graph.

DISCUSSION

We have introduced three different methods for the quantification of the mRNA of the galactose operon in *E. coli*. Quantification of the total mRNA can be easily achieved with ordinary real-time qPCR. However, quantification of mRNAs that are only 5 bases different in their 5′ termini could not be easily achieved by any conventional methods of quantification. Though the methods reported in this study yielded relative quantities of *P1*-and *P2*-transcripts that are consistent with one and other, it is possible that these methods would yield problems that could surface under different experimental conditions.

The RNA ligation step during the RACE assay seems to be problematic especially when an experimenter wants to measure the *gal* mRNA concentration in low abundance. The ligation efficiency can be estimated by the comparison of the Ct values of the *P2*-transcript in the RACE real-time qPCR assay (ligated) with that in the real-time qPCR only assay (non-ligated). These were around 26 in the ligated, and 18 in the non-ligated (55.3°C in Fig. 4B), suggesting that the ligation efficiency of the 5S rRNA to the *gal* mRNA is roughly $1/2^8$ or 1/256. Thus, measuring the *gal* mRNA in a repressed condition could possibly yield a variation in quantity in between samples greater than the actual changes.

The PCR amplification step during RACE could become problematic due to the well known drawback of PCR: the end point reaction (13). Since amplification of the *P1*-and *P2*-transcripts occurs simultaneously in a single tube during the RACE assay, we considered that the quantitative measurements of the resulting gel using band densitometry, such as PhosphorImager™ (Fig. 3A), could reveal the ratio of the two reliably. However, for the questions whose answers require quantitative comparisons between the samples (such as how much *P1* transcripts increases or decreases at different times of

growth) we considered, real-time qPCR would be a better method than the RACE assay, because the RACE result is based on an end-point PCR reaction. The concentration range of cDNA showing the linear response in the amplification reaction of real-time qPCR is 4 to 5 orders of magnitude more than that of the end-point PCR reaction (13).

Considering these problematic steps in RACE for quantification of the *gal* mRNA, we suggest that the real-time qPCR with the P2only-for primer would be the choice for quantification of the galactose-operon mRNAs which differ by 5 bases in their 5′-ends.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The bacterial strains used in this study were MG1655, MG1655 *kan*, MG1655 *kan* $P1^+P2^-$, and MG1655 *kan* $P1^-P2^+$. Generation of MG1655 *kan*, MG1655 *kan* $P1^+P2^-$, and MG 1655 *kan* $P1^-P2^+$ has been described (14, 15). Strains were grown to an OD_{600} of 0.6 in LB containing 0.5% galactose at 37°C.

RNA preparation

Early log phase cells (about 2×10^8 cells at an OD_{600} of 0.6) were immediately harvested and resuspended in 50 μl of protoplasting buffer (15 mM Tris-HCl [pH 8.0], 0.45 M sucrose, 8 mM EDTA). Lysozyme (5 μl of 50 mg/ml) was added, and the sample was incubated for 5 min at 25°C. A phenolic detergent (1 ml, TRIREAGENT, Molecular Research Center, USA) was added, and then the sample was mixed by vortexing for 10 sec and incubated for 5 min at 25°C. Chloroform (200 μl, Sigma, USA) was added, and the mixture was vortexed vigorously for 20 sec, and then incubated for 10 min at 25°C. The resulting mixture was centrifuged at 10,000 × g for 15 min at 4°C. The aqueous phase (500 μl) was transferred to a new tube and mixed with 500 μl of isopropanol (Sigma). The sample was mixed and incubated for 10 min at 25°C. RNA was collected by centrifugation at 10,000 × g for 15 min at 4°C and washed with 1 ml of 75% cold ethanol. The precipitated RNA was dissolved in 50 μl of RNA storage buffer (Ambion, USA). Genomic DNA in the RNA sample was removed with Turbo DNA-free™ (Ambion) as the manufacturer recommended. Concentration of the RNA was determined by measuring the absorbance at 260 nm using a NanoDrop™ spectrophotometer (Thermo Scientific, USA).

Rapid amplification of cDNA 5′-Ends (5′ RACE) of the *gal* mRNA

The 5′-RACE assay was initiated with treatment of the total RNA with tobacco acid pyrophosphatase (Epicentre, USA). The reaction was set up in 25 μl, in which 5 μg of total RNA, 15 units of the enzyme, and 40 units of RNasin (Promega, USA) were incubated at 37°C for 2 h. RNA ligation on the pyrophosphatase-treated RNA was performed at 37°C for 3 h in 25 μl containing 12.5 μl of RNA, 2.5 μl of 10X reaction buffer (Ambion), 10 units of T4 RNA ligase (Ambion) and 20 units of RNasin. The ligated RNA was purified using a G-50 column (Amersham Biosciences, UK). This RNA ligation reaction was intended to ligate the 3′-hydroxyl end of the 5S rRNA to the 5′-phosphate end of mRNA. cDNA from this RNA preparation was generated using a random hexamer. RNA (13.75 μl) from the ligation reaction was reverse transcribed at 37°C for 3 h in a 20 μl reaction volume containing 4 units of Omniscript Reverse Transcriptase (Qiagen, Germany), 0.5 mM of each dNTP, 10 μM random hexamer (Takara, Japan), and 10 units of RNasin. The *gal*-specific cDNA was amplified with a *galE*-specific DNA primer (E2, 5′-TGGGCTTTTTGCAGATCGGTGA GGA-3′) and 5SF primer (5′-CCATGCGAGAGTAGGGAACTG CCA-3′) complementary to the 5S rRNA that could hybridize to the 3′ end of 5S rRNA that is now at the 5′ end of the cDNA, under the following conditions: an initial denaturation at 95°C for 5 min; 30 cycles of 30 sec of denaturation at 94°C, 30 sec of hybridization at 60°C and 40 sec of elongation at 72°C; 5 min of additional elongation at 72°C. The amplified cDNA was purified using a QIAQUICK® PCR Purification Kit (Qiagen). The cDNA was eluted in 50 μl of elution buffer (Qiagen), and used in the following assay.

Primer extension reaction

Purified cDNA (10 μl) prepared in the RACE assay above was used as a template for the primer extension reaction performed in 20 μl, containing a ^{32}P-labeled primer (complementary to *galE* mRNA, 5′-ACCACCGGTAACCAGAACTC-3′), 0.5 mM of each dNTP, and 1 unit of HotStarTaq™ DNA polymerase, under the following conditions: an initial denaturation at 95°C for 5 min; 25 cycles of 30 sec of denaturation at 94°C, 90 sec of hybridization at 52°C and 20 sec of elongation at 72°C. The reaction products were resolved on an 8% polyacrylamide-urea sequencing gel and quantified using a PhosphorImager (Bio-Rad, USA).

Real-time qPCR

The template cDNA for real-time qPCR was prepared as described for the 5′ RACE assay above. PCR primer sets using real-time qPCR were designed by Primer 3 software (16). It was optimized and confirmed that all PCR primer sets had same amplification efficiencies. Real-time qPCR (CFX96™, Bio-Rad, USA) was performed in a 20 μl reaction containing 10 μl of iQ™ SYBR® Green Supermix (Bio-Rad), 7.2 μl of nuclease-free water, 0.4 μl each of forward primer (10 mM) and reverse primer (10 mM), and 2 μl of the cDNA template, under the following conditions: an initial denaturation step at 94°C for 3 min; 40 cycles of 15 sec of denaturation at 94°C, 20 sec of hybridization at 60°C and 15 sec of elongation at 72°C.

RNase protection assay

The RNase protection assay was performed using RPA III™ kit (Ambion). A PCR-DNA fragment of 130 bp containing the pro-

moter region of the *gal* operon (bases from −5 to 125) was cloned to pGEM-T-Easy Vector (Promega). The resulting plasmid was linearized and used as a template for an *in vitro* transcription of the cloned *gal* promoter region. Anti-sense RNA with 226 nucleotides was transcribed using T7 RNA polymerase in the presence of [α-^{32}P] UTP (Amersham Biosciences). The radio-labeled probe was hybridized to 10 μg of the total RNA in a 10 μl hybridization III buffer (Ambion). After overnight hybridization at 42°C, 150 μl of an RNaseA and RNaseT1 mixture (250 and 10,000 units/ml, respectively) was added. This reaction was continued at 37°C for 30 min, and protected RNA fragments were collected by isopropyl alcohol precipitation. The protected RNA fragments were resolved on 8% polyacrylamide-urea sequencing *gel* and visualized by autoradiography. Quantification of the RNA bands was performed using PhosphorImager™.

Acknowledgements
This work was supported by the Korea Science and Engineering Foundation (KOSEF) Grant funded by the Korea government (MEST R01-2006-000-10902-0) and by the National Research Foundation of Korea Grant (NRF-2008-2581).

REFERENCES

1. Adhya, S. and Miller, W. (1979). Modulation of the two promoters of the galactose operon of *Escherichia coli*. *Nature* **279**, 492-494.
2. Musso, R. E., Di Lauro, R., Adhya, S. and de Crombrugghe, B. (1977). Dual control for transcription of the galactose operon by cyclic AMP and its receptor protein at two interspersed promoters. *Cell* **12**, 847-854.
3. Geanacopoulos, M., Vasmatzis, G., Zhurkin, V. B. and Adhya, S. (2001). Gal repressosome contains an antiparallel DNA loop. *Nat. Struct. Biol.* **8**, 432-436.
4. Lewis, D. E. and Adhya, S. (2002). *In vitro* repression of the *gal* promoters by GalR and HU depends on the proper helical phasing of the two operators. *J. Biol. Chem.* **277**, 2498-2504.
5. Aki, T. and Adhya, S. (1997). Repressor induced site-specific binding of HU for transcriptional regulation. *EMBO J.* **16**, 3666-3674.
6. Semsey, S., Virnik, K. and Adhya, S. (2006). Three-stage regulation of the amphibolic gal operon: from repressosome to GalR-free DNA. *J. Mol. Biol.* **358**, 355-363.
7. Semsey, S., Krishna, S., Sneppen, K. and Adhya, S. (2007). Signal integration in the galactose network of *Escherichia coli*. *Mol. Microbiol.* **65**, 465-476.
8. Heid, C. A., Stevens, J., Livak, K. J. and Williams, P. M. (1996). Real time quantitative PCR. *Genome Res.* **6**, 986-994.
9. Frohman, M. A., Dush, M. K. and Martin, G. R. (1988). Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. *Proc. Natl. Acad. Sci. U.S.A.* **85**, 8998-9002.
10. Bingham, A. H., Ponnambalam, S., Chan, B. and Busby, S. (1986). Mutations that reduce expression from the *P2* promoter of the *Escherichia coli* galactose operon. *Gene* **41**, 67-74.
11. Dreyfus, M., Kotlarz, D. and Busby, S. (1985). Point mutations that affect translation initiation in the *Escherichia coli galE* gene. *J. Mol. Biol.* **182**, 411-417.
12. Lee, J. J. and Costlow, N. A. (1987). A molecular titration assay to measure transcript prevalence levels. *Methods Enzymol.* **152**, 633-648.
13. Schmittgen, T. D., Zakrajsek, B. A., Mills, A. G., Gorn, V., Singer, M. J. and Reed, M. W. (2000). Quantitative reverse transcription-polymerase chain reaction to study mRNA decay: comparison of endpoint and real-time methods. *Anal. Biochem.* **285**, 194-204.
14. Datsenko, K. A. and Wanner, B. L. (2000). One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 6640-6645.
15. Lee, H. J., Jeon, H. J., Ji, S. C., Yun, S. H. and Lim, H. M. (2008). Establishment of an mRNA gradient depends on the promoter: an investigation of polarity in gene expression. *J. Mol. Biol.* **378**, 318-327.
16. Rozen, S. and Skaletsky, H. (2000). Primer3 on the WWW for general users and for biologist programmers. *Methods Mol. Biol.* **132**, 365-386.