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Effects of Glucose Repression and Plasmid Copy Number on Cloned Gene Expression in Recombinant Yeast

Eock-Kee Hong
Department of Fermentation Engineering
College of Engineering, Kangwon National University, Chunchon 200-701, Korea

재조합 효모에서의 포도당 억제와 Plasmid 수가 유전자 발현에 미치는 영향

홍 억 기
강원대학교 공과대학 발효공학과

ABSTRACT

Deletions between UAS_G and the *GAL1* TATA box reduced glucose repression and allowed constitutive expression of the gene product in the absence of galactose. The relative inducer level (ratio of galactose/glucose concentrations) affected the extent of gene expression and glucose repression. Glucose repression was reduced by a factor of 2 to 5 as the relative inducer level increased. In the medium containing galactose only, induction of β-galactosidase synthesis by galactose increased with plasmid copy number. On the contrary, plasmid copy number didnot affect significantly β-galactosidase synthesis in the medium containing both glucose and galactose (2% glucose+2% galactose), which might be due to glucose repression caused by high glucose concentraion. However, when the medium contained the relatively high inducer level (0.4% glucose+0.8% galactose), β-galactosidase synthesis increased with plasmid copy number, indicating that the beneficial effect of higher galactose concentration was weaker than the repressive effect of higher glucose concentration.

INTRODUCTION

In galactose catabolism, the *GAL4* protein specifically binds to an upstream activation sequence (UAS_G), which is positioned between *GAL1* and *GAL10* genes, and activates transcriptions of the necessary genes for galactose catabolism (*GAL1*, *GAL7*, and *GAL10*) (1-3). The activity of *GAL4* protein is inhibited by the negative regulator, *GAL80* protein, when cells are grown in the absence of galactose. *GAL80* prevents transcription from the *GAL* promoter by binding to *GAL4*. This inhibition can be overcome by the addition of galactose which interacts with the repressor, *GAL80*, resulting in releasing the *GAL4* protein (4-6). Thus, gene expression from a *GAL* promoter, which is induced by galactose, can be increased by increasing the concentration of *GAL4* and decreasing the concentration of *GAL80* (7).

In addition, glucose represses tanscription of the *GAL* genes by inhibition of the binding of *GAL4* protein to UAS_G (2). Yeast cells contai-

ning a *GAL1–lacZ* fusion plasmid synthesize β-galactosidase only in the presence of galactose, and this synthesis is inhibited by glucose (6). Matsumoto et al. (8) showed that, for comparison, galactokinase (*GAL1*) is induced 1000-fold by galactose. Whenever cloned gene expression is observed, it is dependent on galactose and repressed by glucose. That is, increased relative inducer levels (i. e., the ratio of galactose/glucose concentration) could reduce glucose repression. Deletions between UAS_G and the *GAL1* TATA box allow β-galactosidase to be synthesized in the absence of galactose even though at low levels, indicating constitutive gene expression. These deletions seem to reduce the repression caused by glucose. In this work, the various concentration ratios of glucose and galactose were applied to study the effect of glucose repression on β-galactosidase synthesis.

Cloned gene expression generally depends on the plasmid stability and the copy number. A plasmid exhibiting higher stability leads to high levels of gene expression. If the stability is maintained at a high level, most of the population can produce the cloned gene product. On the other hand, when the stability is low, only a small fraction of the population can produce the cloned gene product. In non-selective media, the plasmid -free cells will become rapidly dominant in the population. Since plasmid-free cells, even in the selective medium, can grow for several generations until the plasmid-encoded proteins for selection are degraded or diluted to the point that they become growth limiting (9–11), stability should be maintained at a high level to yield high cloned gene productivity. Plasmid copy number also plays an important role in gene expression since copy number can affect the stability of the vector plasmid (12, 13). High copy number yields high expression levels, but this effects could be often masked by glucose repression. Thus, it is desirable to reduce glucose repression and to maintain high plasmid stability and copy number in order to enhance cloned gene expression. In this work, the effect of plasmid copy number on cloned gene expression was studied in the presence of glucose repression.

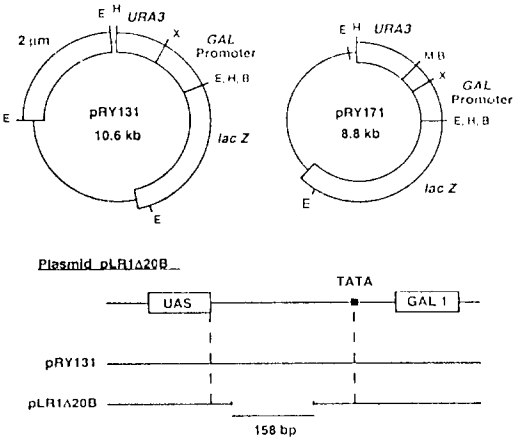

Fig. 1. General structures of plasmids pRY131, pRY171, and pLR1△20B. Plasmid pRY171 is essentially the same as plasmid pRY131 except lacking 2μm sequences. Plasmid pLR1△20B is exactly the same as plasmid pRY131 except for the deletions between UAS_G and the *GAL1* TATA box. Restriction sites: B, *Bam*HI; E, *Eco*RI; H, *Hind*III; M, *Mal*I; X. *Xba*I.

MATERIALS AND METHODS

Strains and Plasmids

Yeast strains were *Saccharomyces cerevisiae* S150-2B[cir$^+$] and [cir^0] (*MATa leu2-3 leu2-112 his3 ura3-52 trp1*). These are isogenic yeast strains containing and lacking the endogenous 2 μm circle plasmids, respectively. Yeast strains were kindly supplied by J. R. Broach (Princeton University, Princeton, NJ). Plasmids pRY131, pLR1△20B, and pRY171 have similar constructions (Fig. 1). Plasmid pRY131 is derived from plasmid pRY121(6). Plasmid pRY131 has the *lacZ* (β-galactosidase) gene fused at the *GAL1* site of the *GAL1–GAL10* hybrid promoter which controls the transcription of the *lacZ* gene. This plasmid contains an *Eco*R1 fragment including the origin of replication and the *REP3* locus from

the endogenous 2㎛ circle plasmid. Plasmid pLR1 △LR20B is exactly the same as plasmid pRY131 except for deletions between UAS_G (*GAL* upstream activating sequence) and the *GAL1* TATA box. These deletions which are 158 base pairs long, allow constitutive synthesis of cloned gene product and reduce glucose repression (14). Plasmid pRY171 is essentially similar to plasmid pRY131. This plasmid has a *GAL1* promoter, but doesnot contain the 2㎛ sequences. That is pRY171 doesnot have the origin of replication, hence, this plasmid should be integrated into the chromosome in order to be replicated (6). All of the above three plasmids have a *URA3* gene providing a selective advantage to plasmid-harboring cells. All of the plasmids used in this work were kindly supplied by M. Ptashne (Harvard University, Cambridge, MA).

Medium

The medium for yeast strains was SD minimal medium containing bacto-yeast nitrogen base without amino acids (6.7g/ℓ), L-histidine (100mg/ℓ), leucine (150mg/ℓ), L-tryptophan (100mg/ℓ), and glucose (20 or 4g/ℓ) and/or galactose (20 or 8g/ℓ). Concentrations of glucose (4g/ℓ) and galactose (8g/ℓ) were determined for the C-limited growth medium and for the full-induction of β-galactosidase synthesis, respectively. Galactose was added freshly just before starting cultivation. All of SD minimal media were buffered to pH 5.5 with 0.1M citrate buffer.

Culture Conditions

Erlenmyer flasks (250ml) containing 20ml of selective SD medium (*ura-*) were inoculated from a single colony and incubated in a shaker for 20-24 hours. Flasks containing 50ml of fresh SD medium were inoculated at an inoculum level of 1% (v/v) and incubated in a gyrotory shaker (Model G25, New Brunswick Scientific) at 250rpm and 30℃. Samples were taken at ca. 50 hours of culturing time to measure β-galactosidase specific activity at the late-exponential growth phase.

General Procedures

Yeast transformation and plasmid DNA isolation were performed as previously described by Sherman et al. (15). β-galactosidase assays were performed as described by Miller (16) with the modification of West et al. (14). An aliquot of cell suspension was diluted in Z buffer (pH 7) to give a total volume of 1ml. The cells were lysed by the addition of 100㎕ of chloroform and 50㎕ of 0.1% SDS. At 28℃ the reaction was started by adding 200㎕ of ONPG (*O*-nitrophenyl-β-D-galactopyranoside, 10mg/ml) and stopped by adding 500㎕ of 1M Na_2CO_3 after sufficient yellow color had developed. The optial density was measured at both 420nm and 550nm by a spectrophotometer (Baush & Lomb, Model Spectronic 601). β-galactosidase specific activity (units/min/g cells) was determined by normalizing the activity by the mass of cells. Plasmid copy number was measured as described by Broach (17).

RESULTS AND DISCUSSION

Glucose repression appears to be a negative effect on the cloned gene expression in glucose-containing medium. Glucose repression can be reduced by genetic manipulation and inducer level. Deletions between the loci UAS_G and the *GAL1* TATA box leaving both sequences intact reduce glucose repression and allow constitutive expression of the gene product in the absence of galactose (14), i.e., when glucose is a sole carbon-source in the medium. The inducer (galactose) level is also important for the cloned gene expression by changing the ratio of galactose/glucose concentrations (6, 18).

β-galactosidase specific activity at various combinations of glucose and galactose concentration is shown in Table 1. β-galactosidase specific activity was measured at ca. 50 hours of culture time to study the effect of glucose repression on the cloned gene expression at the late-exponential grwoth phase. When there was no galactose in the medium, only strain S150-2B[cir^+]:pLR1 △20B could produce β-galactosidase without galactose induction, even though at low levels, as shown in Table 1. This is due to the deletions (158 bp) between UAS_G and the *GAL1* TATA

Table 1. β-galactosidase specific activity[a] in *S. cerevisiae* S150-2B grown in minimal selective media.

Strain	2% Glu	2% Glu + 2% Gal	2% Gal	0.4% Glu	0.4% Glu + 0.8% Gal	0.8% Gal
S150-2B[cir$^+$]:pLR1△20B	254	905	8724	274	1925	7086
S150-2B[cir$^+$]:pRY131	0	341	8945	0	1243	6908
S150-2B[cir^0]:pRY131	0	275	4058	0	533	3298
S150-2B[cir^0]:pRY171	0	211	1848	0	360	1746

[a]In [units/min/g cells]

box, which reduce the glucose repression. These deletions seemed to cause a constitutive synthesis of β-galactosidase as well as significantly less repression by glucose, even though there was no inducer (galactose) in medium. If the medium included galactose only, β-galactosidase activity was very high in each strain. There was no glucose repression which inhibited the cloned gen expression. For all strains, β-galactosidase activities were somewhat higher in 2% galactose-containing medium than in 0.8% galactose-containing medium.

On the other hand, when the medium contained both glucose and galactose (galactose was added at the start of the cultivation), cloned gene expression was severely repressed in all strains tested, i.e., even in the case of strain S150-2B [cir$^+$]: pLR1△20B. In the case of this medium, glucose was completely depleted when β-galactosidase specific activity was measured. On the contrary, galactose concentration was not reduced significantly, indicating that glucose was the primary carbon source. This means that the initial glucose concentration could severely inhibit the cloned gene expression until glucose was depleted. As shown in Table 2, in medium with 2% glucose+2% galactose, the glucose repression ratio (defined as the value of enzyme activity from cells grown on galactose alone divided by the value of enzyme activity from cells grown on glucose plus galactose) (6) is very high. However, in medium with 0.4% glucose+0.8% galactose, the glucose repression is reduced considerably for all strains by a factor of 2 to 5, indicating the production of a fairly high amount of β-galactosidase in the presence of glucose. In Table 1, β-galactosidase specific activity is higher in strain S150-2B[cir$^+$]:pLR1△20B than in strain S150-2B [cir$^+$]: pRY131 by a factor of ca. 3 in medium containing 2% glucose+2% galactose and ca. 2 in medium containing 0.4% glucose+0.8% galactose. Plasmid pLR1△20B is exactly the same as plasmid pRY131 except for the deletions between UAS_G and the *GAL1* TATA box. Hence, it is clear that these deletions reduce glucose repression which inhibits the cloned gene expression. Therefore, glucose repression can be reduced by applying low glucose concentration even though the medium has a low galactose concentration. These results suggest that the relative inducer level (ratio of galactose/glucose concentrations) affects the extent of gene expression and glucose repression. Also, generally, the beneficial effect of higher galactose concentrations is weaker than the repressive effect of higher glucose concentrations. In particular, in the case of strain S150-2B [cir$^+$]:pLR1△20B, the deletions between UAS_G and the *GAL1* TATA box could largely reduce the glucose repression even in the presence of high concentration of glucose in comparison with the other strains.

Strain S150-2B [cir^0]:pRY171 exhibits approximately 100% stability since this plasmid is integrated into the chromosome. The copy number of this plasmid is 1. Strains S150-2B [cir$^+$]:pLR1△ 20B and [cir$^+$]:pRY131 have very high stability. In these strains, there is a *REP* system (which governs plasmid equipartitioning to both progeny cells at cell division) due to the presence of the endogenous 2μm circle plasmids in cells (11, 19),

Table 2. Glucose Repression Ratio[a] in β-galactosidase specific activity.

Strain	2% Glu+2% Gal	0.4% Glu+0.8% Gal
S150-2B [cir$^+$]:pLR1△20B	9.6	3.7
S150-2B [cir$^+$]:pRY131	26.3	5.6
S150-2B [cir^0]:pRY131	14.8	6.2
S150-2B [cir^0]:pRY171	8.7	4.9

[a]Glucose repression ratio is defined as the value of enzyme activity from cells grown on galactose alone divided by the value of enzyme activity from grown on glucose plus galactose.

even though plasmids pLR1△20B and pRY131 do not contain *trans*-acting *REP1* and *REP2* gene sequences required for the *REP* system. The average copy numbers of plasmids pLR1△20B and pRY131 in strain S150-2B [cir$^+$] are about 47 and 44, respectively. On the other hand, the stability of strain S150-2B [cir^0]:pRY131 is very low due to the absence of the *REP* system, even in selective medium. The average copy number of this plasmid is about 5.

Cloned gene expression also depends on the plasmid copy number. Induction of β-galactosidase synthesis by galactose generally increases with plasmid copy number. Fig. 2 and Fig. 3 show the effect of plasmid copy number on the ratio of β-galactosidase specific activity for the three strains to the β-galactosidase specific activity of strain S150-2B [cir^0]:pRY171 which contains a plasmid copy number of 1. In medium containing galactose only, as expected, the ratio of β-galactosidase specific activity increases with plasmid copy number. However, it is observed that the relationship between plasmid copy number and β-galactosidase specific activity is nonlinear. β-galactosidase specific activities of multicopy plasmids are not high as many times that of single-copy plasmid (pRY171) as their plasmid copy numbers. This suggests that there seem to be limiting factors in gene expression. The *GAL4* protein is probably present at only a few copies per cell (6, 20, 21). That is, *GAL4* is limiting and is titrated out by a multicopy plasmid.

Interestingly, plasmid copy number doesnot affect significantly β-galactosidase synthesis in the medium containing both glucose and galactose as shown in Fig. 2. This means that glucose repression severely inhibits the cloned gene expression when the concentration of glucose is high (i.e., relatively low inducere level), even though cells harbor high plasmid copy number. In the case of strain S150-2B [cir$^+$]:pLR1△20B (plasmid copy number of 47), β-galactosidase synthesis is fairly high, showing that the ratio of β-galactosidase

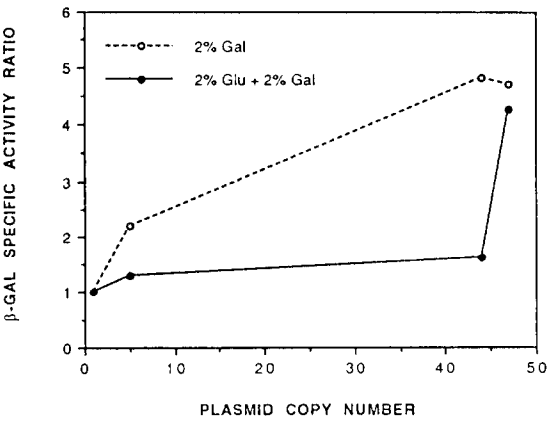

Fig. 2. Effect of plasmid copy number on β-galactosidase specific activity ratio in medium containing 2% glucose+2% galactose.

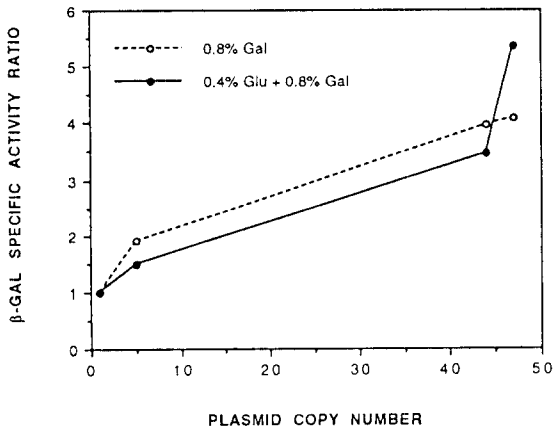

Fig. 3. Effect of plasmid copy number on β-galactosidase specific activity ratio in medium containing 0.4% glucose+0.8% galactose.

specific activity in medium with both glucose and galactose is close to that in medium with galactose only. This is due to the deletions between UAS_G and the *GAL1* TATA box, which reduce the glucose repression.

On the other hand, the β-galactosidase synthesis increases with plasmid copy number as shown in Fig. 3, showing that the increasing rate of the ratio of β-galactosidase specific activity in medium with both glucose and galactose is similar to that in medium with galactose only. Consequently, the difference in the ratio of β-galactosidase specific activity between both medium types is much smaller than that shown in Fig. 2, indicating less glucose repression. In medium with both glucose and galactose, the cloned gene expression increases with plasmid copy number when the medium contains the relatively high inducer level (0.4% glucose+0.8% galactose), whereas plasmid gene dosage doesnot affect significantly the cloned gene expression when the medium contains the relatively low inducer level (2% glucose+2% galactose). These results indicated that the relationship between plasmid copy number and β-galactosidase specific activity depends on the relative inducer levels.

In addition, in the case of strain S150-2B [cir^+]: pLR1△20B, the ratio of β-galactosidase specific activity is higher in medium with both glucose and galactose than that in medium with galactose only as shown in Fig. 3. This is due to the deletions between UAS_G and the *GAL1* TATA box, which reduce glucose repression, resulting in increase of β-galactosidase synthesis. This means that the genetic manipulation plays a very important role in the cloned gene expression in the presence of glucose repression. Therefore, glucose repression can be reduced by relatively high inducer level and genetic manipulation, while the cloned gene expression depends on the plasmid copy number.

요 약

포도당 억제현상은 유전자 조작 및 induder에 의해 감소될 수 있다. UAS_G와 *GAL1* TATA box 사이에서의 유전자 삭제는 포도당 억제현상을 줄이고 갈락토스가 존재하지 않는 조건에서 지속적인 유전자 발현을 도모했다. 상대적 inducer의 양(갈락토스/포도당 농도의 비)은 유전자 발현 및 포도당 억제현상에 영향을 주었다. 포도당 억제현상은 상대적 inducer의 양이 증가함에 따라 2-5배 정도 감소하였다. 또한 유전자 발현은 플라즈미드의 수에 좌우된다. 배지에 갈락토스만 있을 경우 유전자 발현은 플라즈미드의 수가 증가함에 따라 증가하였다. 반면에 배지에 포도당과 갈락토스가 함께 있는 경우 (2% Glu+2% Gal), 플라즈미드의 수는 유전자 발현에 별다른 영향을 주지 못했다. 그러나 높은 상대적 inducer 양이 배지에 있는 경우 (0.4% Glu+0.8% Gal), 플라즈미드의 수가 증가함에 따라 유전자 발현이 증가하였다. 즉, 포도당 억제현상을 줄임으로써 유전자 발현효율을 높이고자 할 때 갈락토스의 농도를 증가시키는 경우보다는 포도당의 농도를 낮춤으로써 상대적 inducer의 양을 높여 유전자 발현을 유도하는 방법이 보다 효율적인 것으로 나타났다.

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