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Construction of Yeast Vectors Potentially Useful for Expression of Eukaryotic Genes as β-galactosidase Fusion Proteins

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Abstract: By both *in vitro* hydroxylamine mutagenesis of the wild type 3-phosphoglycerate kinase gene (PGK) promoter DNA and insertion of the *leu2*-d gene, we have created yeast expression vectors potentially useful for production of eukaryotic genes in yeast. The guanine (G) to adenine (A) change at the -3 position from the ATG start codon of the PGK promoter-based vector rendered a 6~7 times elevated expression of the adjacent eukaryotic gene, and insertion of the *leu2*-d gene in the vector containing the mutated PGK promoter further enhanced the expression of the gene. When expression of the AIDS virus HIV1-*gag*P17 gene in a *lacZ* fusion form was examined with this new vector, a 15 times higher level of expression than that from the original PGK promoter was observed. Northern and Southern analysis showed that this elevated expression is due to the production of a high copy number of mRNA by *leu2*-d gene functioning and by efficient translation of the produced mRNA. Thus, the vector that contained the A at the -3 position from the ATG start codon in the promoter region and the *leu2*-d gene shows increased expression capability and will be potentially useful for production of eukaryotic genes in yeast.

Key words: efficient translation, HIV1 *gag*P17-*lacZ* fusion, plasmid copy number, yeast expression vector.

Expression of eukaryotic genes in yeast has been useful for production of a large quantity of bioactive substances and several yeast promoters have been developed for this purpose. The promoter of the 3-phosphoglycerate kinase gene (PGK) was one of the promoters succ essfully used to clone and express a number of different eukaryotic proteins (Hitzeman *et al.*, 1983a, 1983b; Kingsman *et al.*, 1990). These include immunoglobulin (Wood *et al.*, 1985), interferon-γ (Tuite *et al.*, 1982; Hitzeman *et al.*, 1983b), calf chymosin (Mellor *et al.*, 1983), and HIV antigen (Adams *et al.*, 1987). In some cases, however, expression of foreign genes is not always satisfactory enough to purify the product from yeast cells. Other yeasat promoters, such as alcohol dehydrogenase (ADH), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and chelatin (CUP1) have alternatively been used (Marcreadie *et al.*, 1989; Etcheverry, 1990; Price *et al.*, 1990; Rosenberg *et al.*, 1990). But, it is still necessary to have an improved yeast expression system for the production of eukaryotic proteins in yeast. The primary element to be considered for improved expression of a foreign gene in yeast is to use a strong promoter that contains the enhanced transcriptional efficiency. The second one to be considered is the methods to increase the translational efficiency of the produced mRNA. We have tried to create a new expression vector possessing both of these feature. We report here the construction of the yeast expression vectors that contained the PGK or chelatin promoter with adenine base at the -3 position from the start codon and the *leu2*-d gene. These vectors produce a high copy number of mRNA that can be translated efficiently. Expression of the HIV1-*gag*P17 gene, a tissue specific inhibitor of the methalloproteinase (TIMP) gene, and the human interlukin 6 gene as the *lacZ* fusion proteins were examined with these vectors.

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Materials and Methods

Strains and plasmids

Yeast *Saccharomyces cerevisiae* strains used for expression of the PGK-derived vector was 20B-12 (α,

*trp*1, *pep*4-3), or L3262 (a, *ura*3-52, *leu*2-3,112, *his*4-34). *E. coli* strain was DH5α(F^-, *end*A1, *hsd*R17(r_k^-, M_k^-), *sup*E44, λ^-, *thi*-1, *rec*A1, *gyr*A96, *rel*A1, Δ(*arg*F-*lac*ZYA), u169, ϕ80, *alac*ZΔd15). The plasmids containing wild-type PGK or chelatin promoters were YEpIPT and pCGY1444, and were obtained from Dr. Ron Hitzeman at Genentech Inc. (San Francisco, USA). The plasmid, pSI4 containing the *leu*-2d gene and the plasmid, YEp353 containing the *E. coli lac*Z gene were a generous gift from Dr. James Broach at Princeton University. The plasmid containing HIV1-*gag*P17 coding and linker sequences, pYYM-*gag*1, was described previously (Yoo *et al.*, 1991). The *lac*Z containing plasmid pYH-GZ was constructed from the plasmids YEp 353, YEpIPT and YEP62 as described in a previous report (Yoo *et al.*, 1990).

DNA manipulation and transformation

Plasmid DNAs were prepared from *E. coli* or yeast by the standard methods (Maniatis *et al.*, 1982; Sherman *et al.*, 1986). Yeast transformation followed the lithium acetate method (Ito *et al.*, 1983). To select the clones that contain the modified promoter function, the mutated plasmid DNAs were directly used for yeast transformation. Trp^+ transformants were selected first and then replica plated on X-gal plates. The transformants showing a deep blue color on X-gal plates were selected as the candidate clones containing the altered promoter function. The total nucleic acids were isolated from the deep blue yeast transformants (Sherman *et al.*, 1986) and retransformed into *E. coli*. From the ampicillin-resistant *E. coli* colonies, the plasmid DNAs were isolated and DNA sequences at the promoter regions were determined by the method of dideoxynucleotide (Vieira and Messing, 1982).

Hydroxylamine random mutagenesis of the plasmid DNAs

To introduce random base changes in the promoter region, the chemical mutagensis method described by Rose and Finks (1987) was used. Five μg of the plasmid DNAs were dissolved in cold hydroxylamine solution (0.45 M NaOH, 1 M hydroxylamine-HCl) and incubated for 10~12 h at room temperature. To stop the mutagenesis reaction, 10 μl of 5 M NaCl and 50 μl of 1 mg/ml BSA were added. Then the DNAs were precipitated, washed with ethanol and used for yeast transformation directly. Mutagen reaction time was optimized so that only 4~6 percent of the total transformants became Trp^- transformants.

β-galactosidase assay

The strength of the mutated promoter was compared indirectly by measuring the β-galactosidase activity expressed from the *lac*Z gene fused at the 3′ end of an eukaryotic gene. Yeast transformants expressing a deep blue color on X-gal plates were selected and were grown in YNB media (0.17% yeast nitrogen base without amino acid and ammonium sulfate, 0.5% ammonium sulfate, 2% dextrose, 2% agar for solid medium) until the cell density reached A_{600}=0.6~0.8. Ten ml of the cell culture were then collected and suspended in 1 ml of Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, pH 7.0, 50 mM KCl, 10 mM $MgSO_4$, and 2.7 ml 2-mercaptoethanol/l). Three drops of chloroform and 20 μl of 0.1% SDS were added into the cell suspension and the cell mixture was vortexed for 40 sec. Twenty to one hundred μl of the permeablized cells were used for β-galactosidase assays according to the method of Miller (1982). In the case of the cells containing the chelatin promoter plasmid, the cells were first grown in YNB medium until A_{600} =0.2, and a $CuSO_4$ solution was added to a final concentration of 30 μM to induce expression of the gene. The

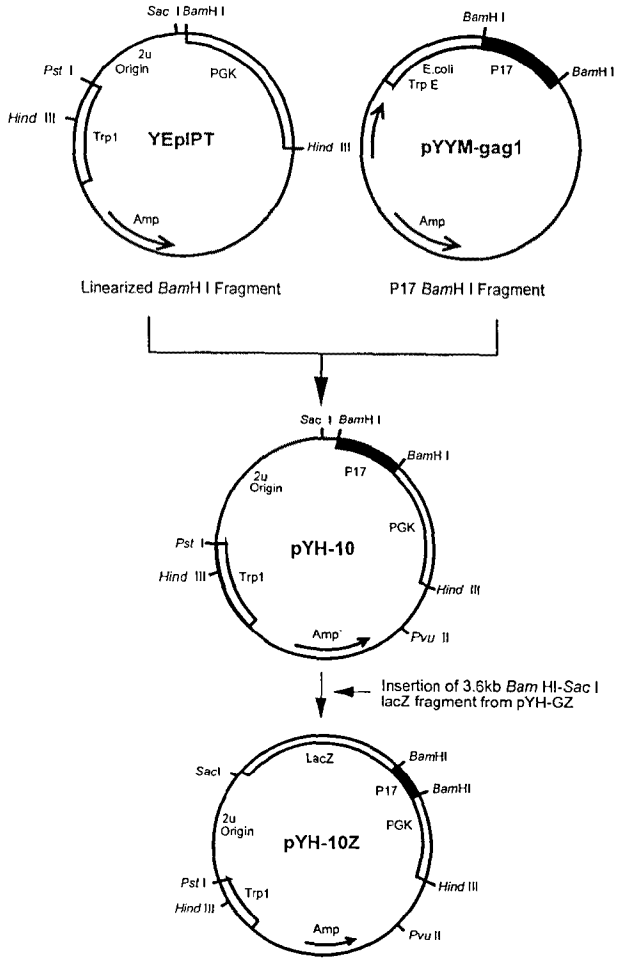

Fig. 1. Construction of the plasmids containing HIV1-*gag*P17 gene under PGK promoter (PYH-10) and *gag*P17-*lacZ* fusion gene (PYH-10Z).

cells were harvested when the cell density reached A_{600} =0.8.

Construction of the plasmid containing HIV1-*gag*P17 gene, *lacZ* gene, and *leu*2-d gene

To test the expression level of a foreign gene easily, the *lacZ*-fusion system was used. As an example of a foreign gene, the HIV1-*gag*P17 gene was first cloned into the plasmid YEpIPT that contained the PGK promoter and the TRP1 selection marker and then the *lacZ* gene of *E. coli* was fused in frame to the *gag*P17 gene (Fig. 1). The 440 bp *Bam*HI fragment containing the *gag*P17 coding and its linker sequences was isolated from the plasmid pYYM-*gag*1 and ligated into the *Bam*HI site of the linear plasmid YEpIPT. The resulting plasmid pYH-10 (Fig. 1) was treated with *Bam*HI and *Sac*I, and the 8.5 kb *Sac*I-*Bam*HI large fragment of pYH-10 was ligated with the 3.6 kb *Bam*HI-*Sac*I fragment of the *lacZ* gene isolated from the plasmid pYH-GZ (Yoo *et al.*, 1990). The resulting plasmid was isolated and the 440 bp *Bam*HI fragment of the *gag*P17 gene was inserted back at the *Bam*HI site. To introduce mutations in the promoter region that leads to increased expression of the adjacent gene, the resulting plasmid DNA pYH-10Z that contained the *gag*P17-*lacZ* fusion gene under the PGK promoter was used as a reference vector and then was mutagenized with hydroxylamine. After transforming the mutated plasmid directly into yeast cells and selecting the colonies showing the highest expression of the *lacZ* gene, the plasmid DNA was isolated and its promoter and coding region sequences were analyzed. The 440 bp *Bam*HI DNA fragment containing only one mutated base right upstream of the ATG codon was isolated from the mutated plasmid DNA and religated into the *Bam*HI-treated linear plasmid pYH-10Z replacing the unmutated 440 bp *Bam*HI fragment. The resulting plasmid, pYH-10ZM, which possesses the PGK promoter with a mutated base right upstream of the ATG codon was then modified further by inserting the *leu*2-d gene (Fig. 2). The 3.7 kb *Hin*dIII-*Pvu*II fragment containing the *leu*2-d gene and the yeast 2 μ replication origin was isolated from the plasmid pSI4, and ligated into the *Hin*dIII-*Pvu*II fragment of pBR322 containing the ampicillin resistant gene and the *E. coli* replication origin (Fig. 2). The resulting 6.2 kb plasmid, pCK-3, was then linearized with *Pvu*II and *Kpn*I and ligated with the 4.8 kb *Dra*I-*Kpn*I fragment of the pYH-10ZM, containing all the PGK promoter sequence necesssary for promoter function, *gag*P17, and *lacZ*. The plasmid, pYH-10Zd, which contained the PGK promoter including the mutated base right upstream of the ATG codon of the *gag*P17, *lacZ*, and the *leu*2-d gene was then tested for expression of the *gag*P17-*lacZ* fusion protein. To test the efficiency of the other promoter function in the presence of the mutated base right upstream of the ATG codon, the PGK promoter sequence upstream of the mutated base was replaced with the chelatin promoter and the resulting plasmid pYH-FZd was tested for its expression of the *gag*P17-*lacZ* fusion protein. In addition, to test the increased expression effected by the modified plasmid on other genes, the plasmids containing the coding region sequence of the tissue-specific inhibitor of the methalloproteinase gene (TIMP) or the human interleukin-6 gene instead of the *gag*P17 gene were constructed.

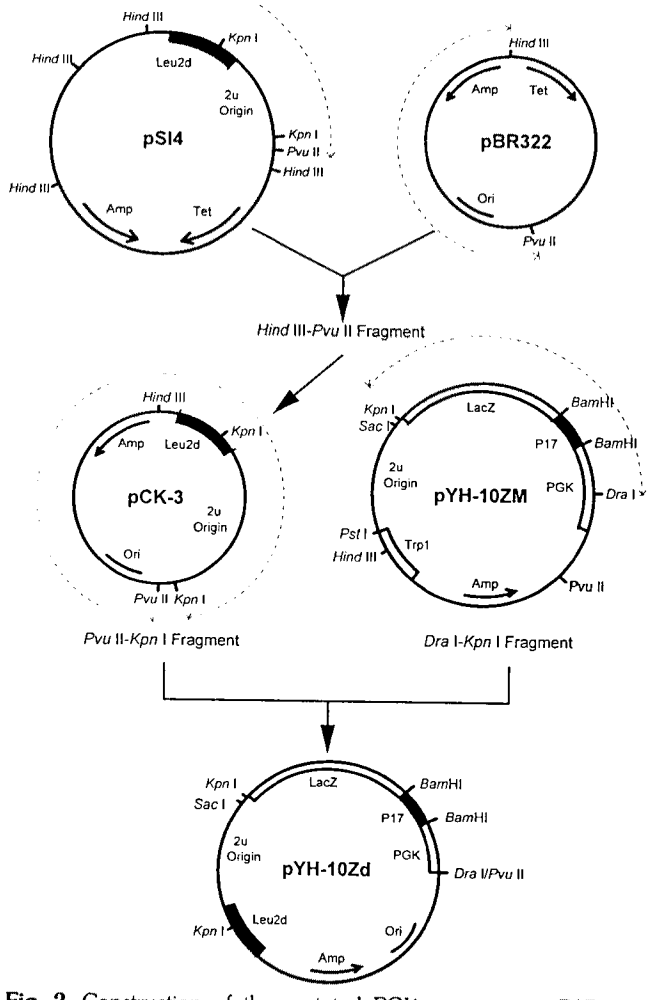

Fig. 2. Construction of the mutated PGK promoter-*gag*P17-*lacZ* fusion plasmid containing the *leu*2-d gene. The plasmid pYH-10 ZM contains the mutated base A instead of G at the -3 position. The sequences around the -3 position of the pYH-10ZM was 5′-GGATCCCAATAGGATGGGT-3′.

SDS-polyacrylamide gel and Western analysis of the expressed *gag*P17-*lacZ* fusion protein

The yeast cells transformed with the PGK or chelatin promoter-based plasmids, pYH-10Z, pYH-10ZM, pYH-

10Zd, pYH-FZ, pYH-FZM or pYH-FZd, were analyzed for their expression of the *gag*P17-*lac*Z fusion protein directly by the SDS-polyacrylamide gel. The transformants were grown in YNB minimal media in the absence of tryptophan or leucine. When cell density reached $A_{600}=0.8$, 10 ml of the cell culture were collected, suspended in 3 ml of the extraction buffer (200 mM Tris HCl, pH 8.0, 10 mM $MgCl_2$, 5 mM EDTA), washed and resuspended in 300 μl of the same buffer. The cell suspension was mixed with the same volume of glass beads, vortexed and centrifuged. The supernatant was mixed with 6X protein sample buffer (0.3 M Tris HCl, pH 6.8, 0.6 M dithiothreitol, 12% SDS, 0.6% bromophenol blue, 60% glycerol), boiled for 2~3 min and analyzed on a 10% SDS-polyacrylamide gel. The gel was stained with Coomassie blue dye. On the other hand, a duplicate gel was electroblotted onto a nitrocellulose paper, treated with the antibody to β-galactosidase, and then with anti-mouse-IgG-alkaline phosphatase conjugate. After washing the filter with TBST buffer (10 mM Tris HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20), 20 ml of alkaline phosphatase buffer (100 mM Tris HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$), 132 μl of NBT and 66 μl of BCIP were added to the filter. The band appearing after a few min was analyzed.

Northern blot analysis of *gag*P17-*lac*Z fusion gene

To measure the amounts of the mRNAs produced from the wild type, mutated, and modified plasmids, total RNAs were prepared from the cells grown in YNB minimal media usng the glass beads-phenol methods (Carlson and Bostein, 1982). Ten μg of the total RNAs were fractionated on a 0.8% agarose-formaldehyde gel, transferred onto nitrocellulose papers and hybridized with the random primed probe DNA of the internal 2.0 kb *Pvu*II fragment of the *lac*Z gene.

Results and Discussion

Isolation of the plasmid possessing increased expression strength of the adjacent gene

In order to develop a yeast expression vector possessing increased strength of expression of a foreign gene in yeast, we used the strategy of modifing the existing yeast promoter sequences by random mutagenesis and increasing the copy number of the gene by inserting the *leu*2-d gene. In addition, to examine the expression level of a foreign gene in yeast easily, the β-galactosidase gene (*lac*Z) of *E. coli* was fused at the 3′ end of a foreign gene as a reporter gene. The plasmid pYH-10Z that contained the HIVI *gag*P17-*lac*Z fusion gene under PGK promoter (Fig. 1) was used as a control plasmid possessing the wild-type promoter strength of PGK. When the plasmid pYH-10Z was mutagenized randomly with hydroxylamine *in vitro*, and used to transform yeast cells directly to select a high expression clone, transformants showing a high level of expression on X-gal indicator plates appeared. Out of 5455 Trp^+ transformants, 421 transformants showed a deep blue color on indicator plates, 604 showed white colonies, and the rest of the transformants showed pale blue. Twelve colonies that were actually shown to have higher β-galactosidase activity than that from unmutated plasmid were selected. Two of these colonies showed the highest level of the β-galactosidase activity (1194 versus the 194 of the pYH-10Z wild-type plasmid). Since these two colonies were expected to contain the plasmid possessing altered expression strength, the DNA sequences of the promoter and coding regions were analyzed.

G to A change at the -3 position from the start codon enhanced the production of the fusion protein

When the promoter and the coding region of the plasmids isolated from the two transformants showing the highest β-galactosidase activity were recloned into the plasmids pTZ18U and pTZ19U and sequenced, only the guanine (G) base at the -3 position from the start codon of *gag*P17 was changed to adenine (A) base. The rest of the promoter and coding sequences were identical. It is likely that A at the -3 position is the preferred base for efficient translation of mRNA, as Kozak has suggested previously (Kozak, 1986). We consider that the increased expression of β-galactosidase by the mutated sequence was due to increased translation of the mRNAs produced under the influence of the PGK promoter.

The *leu*2-d gene together with the adenine base at the -3 position in the noncoding upstream region is potentially useful for the increased expression of a foreign gene in yeast

It is known that the *leu*2-d gene which contains the *S. cerevisiae LEU*2 gene with truncated promoter sequences can complement a *leu*2$^-$ mutant strain by increasing the copy number of the truncated *leu*2 gene in a plasmid, and is shown to be useful for increasing the copy number of the plasmid in the cell (Rose and Broach, 1990). Thus we inserted the truncated *LEU*2 gene, *leu*2-d, in the plasmid pYH-10ZM containing the mutated base at the -3 position. As shown in Fig. 2, the 11 kb plasmid pYH-10Zd was constructed from the *leu*2-d-containing plasmid pSI4 and pYH-10ZM. When the β-galactosidase activity of the cells containing the resulting plasmid pYH-10Zd was examined, the ac-

Table 1. β-galactosidase activities of the modified PGK and chelatin promoter-*lacZ* fusion plasmids containing the *leu*-2d gene

Plasmids (PGK)	β-Gal activity	Plasmids (chelatin)	β-Gal activity
pYH-10Z	196	pYH-FZ	188
pYH-10ZM	1194	pYH-FZM	953
pYH-10Zd	2662	pYH-FZd	2512

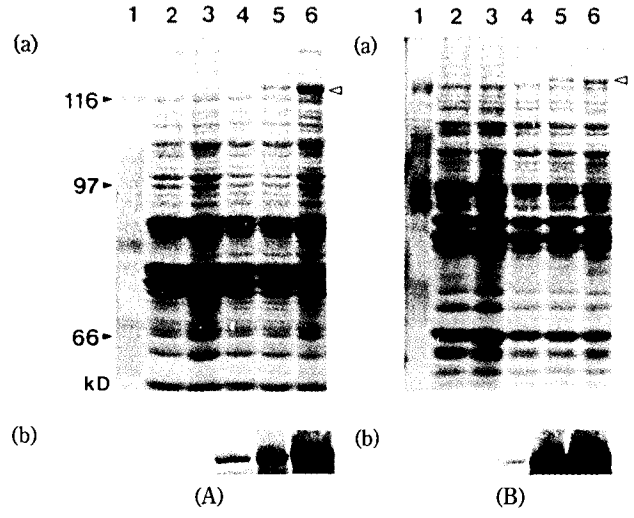

Fig. 3. SDS-polyacrylamide gel and Western analysis of the total yeast protein containing the PGK and chelatin based modified plasmids. A-a) Yeast cell extracts containing the PGK based modified plasmids: lane 1, protein size markers; lane 2, no plasmid (20B-12 strain); lane 3, no plasmid (L3262 strain); lane 4, pYH-10Z; lane 5, pYH-10ZM; and lane 6, pYH-10Zd. B-a) Yeast cell extracts containing the chelatin based modified plasmids: lane 1, protein size markers; lane 2, no plasmid (20B-12 strain); lane 3, no plasmid (L3262 strain); lane 4, pYH-FZ; lane 5, pYH-FZM; and lane 6, pYH-FZd. A,B-b) Western analysis of the upper gels with the β-galactosidase antibody. Open arrows indicate *gag*P17-*lacZ* fusion proteins.

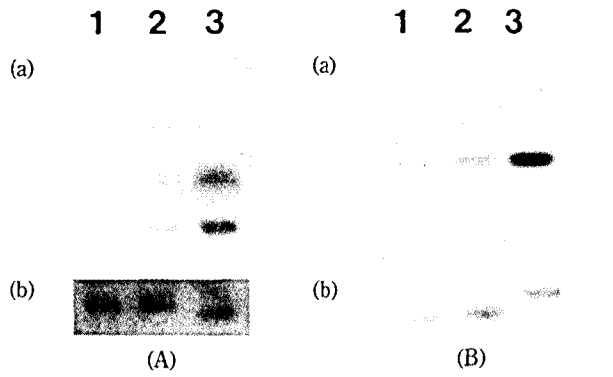

Fig. 4. Northern and Southern analysis of the expressed mRNA and the copy number of the fusion gene. A) The total RNAs in the cells containing the wild-type PGK promoter plasmid pYH-10Z (lane 1), the mutated PGK promoter pYH-10ZM (lane 2), and the *leu*2-d containing plasmid pYH-10Zd (lane 3) were analyzed with the 2.0 kb *lacZ* probe DNA (a) and with the TCM1 probe as an internal control (b) to assess the quantity of total RNA. The two hybridizing bands in (a) may be two different trasnscripts. B) To examine the copy number of the plasmid in the cells, the total DNAs from the same cells containing each plasmid as in A were analyzed with the 2.0 kb *lacZ* probe (a) and with the URA3 probe (b) as an internal control.

tivity was 2~3 or 10~14 times higher than that in the cells containing the mutated promoter plasmid pYH-10ZM or the wild-type PGK promoter plasmid pYH-10Z, respectively (Table 1). Thus the modified plasmid containing the PGK promoter with adenine base at the -3 position and the *leu*2-d gene showed enhanced gene expression strength. SDS-polyacrylamide gel analysis and Western analysis with the antibody to the β-galactosidase confirmed the increased expression of the *gag*P17-*lacZ* fusion protein from both pYH-10ZM and pYH-10Zd (Fig. 3A (a, b)). When the PGK promoter sequences upstream of the -3 position were substituted by chelatin promoter sequences, the same fold of increased expression of the β-galactosidase was observed in pYH-FZ, pYH-FZM, and pYH-FZd (Fig. 3B (a, b) and Table 1). In addition, when the coding sequences of *gag*P17 in the plasmid pYH-10Z, pYH-10ZM, and pYH10-Zd was replaced with the tissue specific inhibitor of the methalloproteinase (TIMP) or the human interleukin-6 gene, the same fold of increased expression was observed (to be published elsewhere). Thus the new plasmid we have constructed through random mutagenesis of the promoter DNA and *leu*2-d insertion was shown to be useful for the increased expression of a gene in yeast.

Northern and Southern analysis of the fusion gene

To test whether the increased expression of the β-galactosidase shown by the mutated sequences at the -3 position and *leu*2-d gene insertion was due to efficient transcription or translation, we examined the mRNA level and the copy number of the plasmid. When the total RNAs prepared from the transformants containing each different plasmid were analyzed by Northern hybridzation, the level of the RNAs in the cells containing the pYH-10ZM was the same as that shown in the cells containing unmutated PGK promoter, pYH-10Z (Fig. 4A-a, lanes 1 and 2). This confirms that the increased expression in the cells containing the mutated sequence at the -3 position is not due to the increased production of mRNA. Since it had been suggested that the adenine base at the -3 position from the start codon is the preferred base for efficient initiation of translation, it is possible that the mutation of the guanine base to the adenine base may have a direct role in increased translation of mRNA to the

*gag*P17-*lacZ* fusion protein. Thus the mutation we had created upstream of the ATG codon does not affect the transcription efficiency of the gene, but it may rather induce more efficient translation of the produced mRNA. On the other hand, in the case of the cells containing the *leu*2-d gene, pYH-10Zd, the level of mRNA increased (Fig. 4A-a, lane 3). We reasoned that the increase of the mRNA is due probably to the increased copy number of the gene itself, as suggested by Rose and Broach (1990). To test this, we did Southern hybridization of the total nucleic acid isolated from the cells containing each plasmid with the same 2.0 kb internal *lacZ* fragment. As shown in Fig. 4B-a, the plasmid copy number increased in the cells containing the plasmid pYH-10Zd (lane 3) while it remained the same in the cells containing the wild-type or mutated sequence plasmid (lanes 1 & 2). Thus we concluded that the increased expression of the *gag*P17-*lacZ* fusion protein from the plasmid containing the adenine base at the -3 position and the *leu*2-d gene was not due to the increased transcriptional efficiency of the promoter itself but due to the increased translation efficiency and total number of the mRNA produced under the influence of the increased copy number of the gene. Thus, our results support the hypothesis that for efficient expression of a gene, it is important to have both an increased number of mRNA molecules either by increasing the copy number of the plasmid or by increasing the promoter function and efficient translation of the produced mRNA. The new plasmid vector we reported here will be an useful one which possesses both of these characteristics.

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References

Adams, S. E., Dawson, K. M., Gull, K., Kingsman, S. M. and Kingaman, A. J. (1987) *Nature* **329**, 68.

Carlson, M. and Botstein, D. (1982) *Cell* **28**, 145.

Etcheverry, T. (1990) *Methods Enzymol.* **185**, 319.

Hitzeman, R. A., Chen, C. Y. and Hagie, F. E. (1983a) *Nucl. Acids Res.* **11**, 2745.

Hitzeman, R. A., Leung, D. W., Perry, L. J., Kohr, W. J., Levine, H. L. and Goeddle, D. V. (1983b) *Science* **219**, 620.

Ito, H., Fukuda, Y., Murada, K. and Kimura, A. (1983) *J. Bacteriol.* **153**, 163.

Kingsman, S. M., Cousens, D., Stanway, C. A., Chambers, A., Wilson, M. and Kingsman, A. J. (1990) *Methods Enzymol.* **185**, 329.

Kozak, M. (1986) *Cell* **44**, 283.

Macreadie, I. G., Jagadish, M. N., Azad, A. A. and Vaughan, P. R. (1989) *Plasmid* **21**, 147.

Maniatis, T., Fritsch, E. F. and Sambrook, J. C. (1982) Molecular Cloning, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Mellor, J., Dobson, M. J., Roberts, N. A., Tuite, M. F., Whites, S., Lowe, P. A., Patel, T., Kingsman, A. J. and Kingsman, S. M. (1983) *Gene* **24**, 1.

Miller, J. M. (1982) Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Price, V. L., Taylor, W. E., Clevenger, W., Worthington, M. and Young, E. T. (1990) *Methods Enzymol.* **185**, 308.

Rose, A. B. and Broach, J. R. (1990) *Methods Enzymol.* **185**, 234.

Rose, M. D. and Finks, G. R. (1987) *Cell* **48**, 1047.

Rosenberg, S., Coit, D. and Tekamp-Olson, P. (1990) *Methods Enzymol.* **185**, 341.

Sherman, F., Fink, G. R. and Hicks J. B. (1986) Method in Yeast Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Tuite, M. F., Dobson, M. J., Roberts, N. A., King, R. M., Burke, D. C., Kingsman, S. M. and Kingsman, A. J. (1982) *EMBO J.* **1**, 603.

Vieira, J. and Messing, J. (1982) *Gene* **19**, 259.

Wood, C. R., Boss, M. A., Kenton, J. H., Calvert, J. E., Roberts, N. A. and Emtage, J. S. (1985) *Nature* **314**, 446.

Yoo, H. S., Choi, W. J., Lee, H. W., Kim, K. M., Kwon, J. B. and Cooper, T. (1990) Reserch Report of KIST BSF 80230-250-1, The Ministry of Science and Technology of Korea.

Yoo, H. S., Jang, W. H., Park, H. D., Hyun, S. W., Nham, S. U. and Lee, Y. I. (1991) *Korean J. Microbiol.* **29**, 1.