0273-2289/97/6602—0147$10.75

Construction and Characterization of A Biotin-Regulated Gene Expression System in *Escherichia coli*

YUO-SHENG CHANG AND DAVID SHIUAN*

Department of Biology and Institute of Life Sciences, National Sun Yat-Sen University, Kaohsiung, Taiwan, ROC

ABSTRACT

An autoregulated gene expression system in *Escherichia coli* was designed such that the cloned genes on the vector were not expressed until biotin was depleted during cell growth. The expression vectors were constructed by assembling the DNA fragments containing the regulatory region of the *E. coli* biotin operon (*bio* operon), the universal ribosome-binding site (RBS) and the strong transcription terminator rrnBT_1T_2. The promoter region was further modified by site-directed mutagenesis to create promoters of varied strength. The feasibility of this system was examined in *E. coli* strain R901 (with *bio* operon deleted) using various marker genes, including the *E. coli birA* gene, T7 RNA polymerase gene and yellowfin-porgy growth-hormone gene. The results demonstrated that the induction of marker-gene expression can be triggered as the biotin concentration drops to a threshold value of approximately 2 ng/mL by metabolic utilization.

Index Entries: Recombinant DNA; biotin; regulation; gene expression system.

Abbreviation: *bio* operon, biotin operon; bp, base pair(s); kb, kilo base-pair(s); nt, nucleotide(s); *o*, operator; RBS, ribosome-binding site; T-broth, trypone broth.

INTRODUCTION

One of the major strengths of modern recombinant DNA technology is its ability to produce large quantities of heterologous proteins for

*Author to whom all correspondence and reprint requests should be addressed.

research and pharmaceutical purposes *(1,2)*. Heterologous genes are usually induced to very high levels in the four major gene expression systems, namely *Escherichia coli (E. coli)*, bacillus, yeast and mammalian cells *(3,4)*. Among them, *E. coli* is often the desirable host for a large number of expression needs *(5,6)*. Many stable, high-copy-number plasmids have been described to achieve high-level expression of heterologous proteins. However, continuous proteolysis of the heterologous proteins, especially in the heat-shock-induced gene-expression systems *(7)*, could present a problem. In addition, some heterologous proteins might be detrimental to *E. coli* once they accumulated in the cell, thus making the situation even more difficult. Therefore, the inducibility or the controllability *(8–12)* of the expression system becomes very critical to assure high levels of gene expression and stable-gene products.

In the present study, we have designed an autoregulated gene-expression vector based on the repressor-operator interaction of the *bio* operon coupled to an *E. coli* host cell with its *bio* operon deleted *(13)*. The autoregulated system can thus be turned on by the depletion of the added biotin during cell growth. The expression vectors were constructed by assembling the DNA fragments containing the regulatory region of *E. coli bio* operon *(14,15)*, the universal ribosome-binding region and the strong transcription terminator rrnBT_1T_2 *(16)*. The promoter region was further modified by site-directed mutagenesis to create promoters of varied strength in both orientations. The feasibility of this system was examined using various marker genes including the *E. coli birA* gene *(17)*, T7 RNA polymerase gene *(6)* and the yellowfin-porgy growth-hormone gene *(18)*. The results demonstrated that the expression of marker genes was repressed initially as medium biotin concentration was high, and its induction could be triggered as the biotin concentration dropped to a threshold value of approximately 1 ng/mL by the metabolic utilization during cell growth. The current system not only provides efficient control of heterologous gene expression, but also avoids many disadvantages of previously described systems which rely on heat shock or added chemical inducers to turn on gene expression *(7)*.

METHODS

The bacterial strains and plasmids used in the present study are listed in Table 1.

Construction of the pUC, pUS, and pUT Series-Expression Vectors

The key element of the expression vector, the promoter-operator overlapping region, is derived from the *E. coli bio* operon *(19,20)*. As shown in Fig. 1, the 165-bp *Sau*3A fragment (from nt –40 to +125) of plasmid pUC1A containing the 1.3-kb *bio'AoB'* sequence, *Xho*II fragment *(15)* was subcloned into the *Bam*HI site of vector pUC18, thereby generating plas-

Table 1
Strains and Plasmids

Strain and plasmid	Relevant Genotype	Source or reference
R901	Sm^RΔ*bio A–D*	Cleary and Campbell *(13)*
JM107	F*traD36 lac*I^q(Δ*lacZ)M15 proAB*Δ *(lac-proAB) thi gyrA96* ($Na1^r$) *endA1 hsdR17* ($r_K^-m_K^+$) *relA1 supE44 mcrA*	Yanisch-Perron et al. *(22)*
CJ236	F-*dut ung1 thi-1 relA1*/ pCJ105 Cm^R	Kunkel *(21)*
DH5α	F *endA1 hsdR17* ($r_K^-m_K^+$ *supE44 thi-1 relA1 gyrA96* ($Na1^r$) Δ*(lacZYA-argF)U169* (ϕ*80d lac*Δ*(lacZ)M15))*	Hanahan et al. *(27)*
DG116	λc*I*857George *(7)*	
pUC18, pUC19	*lac*$^+$ Ap^R	Vieira and Messing *(28)*
pG308N	λP_L	Lin et al. *(17)*
M13mp18/mp19	*lacZ*	Yanisch-Perron et al. *(22)*
pUC11A	pUC18::*bio'AoB'* (wild-type)	Shiuan and Campbell *(15)*
pUC98A	pUC18::*bio'AoB'* (P_{98} mutant)	this work
pUC99A	pUC18::*bio'AoB'* (P_{99} mutant)	this work
pUS11A	pUC11A::RBS	this work
pUS98A	pUC98A::RBS	this work
pUS99A	pUC99A::RBS	this work
pUT98A	pUS98A::rnBT_1T_2	this work
pUT99A	pUS99A::rnBT_1T_2	this work
pUS11A-1	pUS11A::*birA*	this work
pUS98A-1	pUS98A::*birA*	this work
pUT98A-1	pUT98A::*birA*	this work
pUT99A-1	pUT99A::*birA*	this work
pUS11A-2	pUS11A::T7 *RNA pol*	this work
pUT98A-2	pUT98A::T7 *RNA pol*	this work
pUS11A-3	pUS11A::ypGH gene	this work
pUT98A-3	pUT98A::ypGH gene	this work
pUT99A-3	pUT99A::ypGH gene	this work
pDSC126	pUC19::$P_{\alpha amy}$::T7 *RNA pol*	this laboratory
pypGH	pUC18::ypGH gene	Tsai et al. *(18)*
pBIRA308N	pG308N::*birA*	Lin et al. *(17)*
pBIRA18	pUC18::*birA*	this work

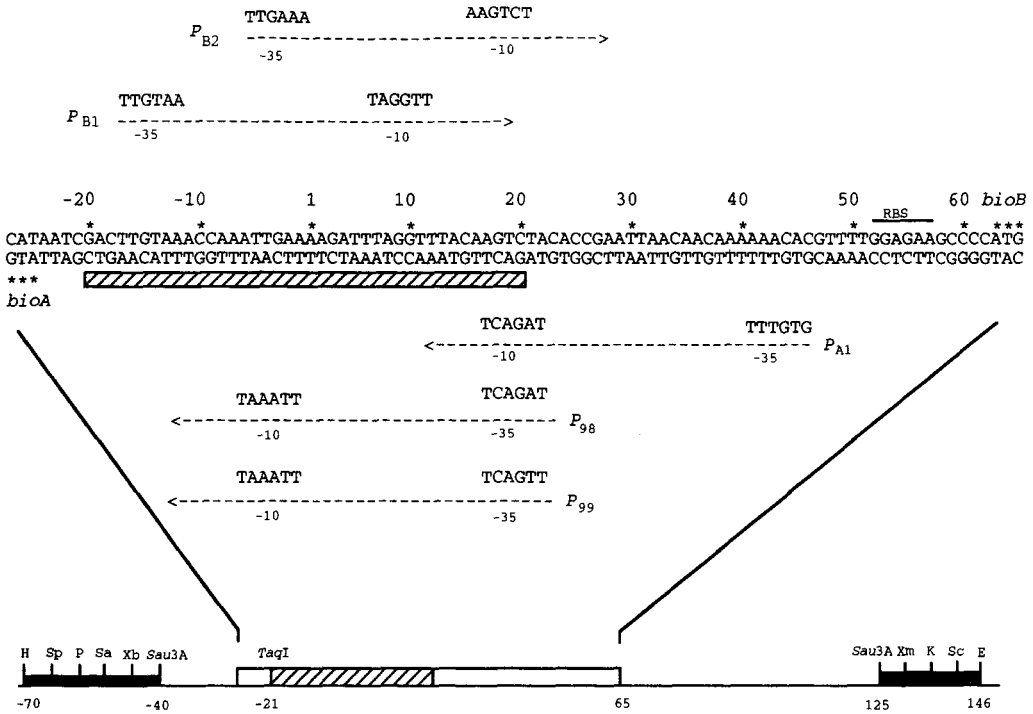

Fig. 1. Regulatory sequence derived from the *E. coli bio* operon. The 165-bp *Sau*3A fragment of plasmid pUC1A (containing the 1.3-kb *bio'AoB'* sequence, *Xho*II fragment) was subcloned into the *Bam*HI site of vector pUC18 to obtain plasmid pUC11A. The 216-bp *Eco*RI-*Hin*dIII fragment was then subcloned into M13mp18 to perform site-directed mutagenesis *(21)*. The mutations are of three categories: insertion of a T between nt –18 and –19 to create a *Cla*I site (5'-ATCG AT-3'); a GC to TA mutation at nt –3, to create a better –10 region, 5'-TTAAAT-3', of a strong leftward promoter P_{98}; and a GC to TA mutation at nt –3, a TA to AT mutation at nt +21, to create a consensus –35 region, 5'-TTGACT-3', a potentially stronger leftward promoter P_{99}. The original leftward promoter P_{A1} and two rightward promoter P_{B1} and P_{B2} with their –35 and –10 regions are also indicated. The dashed box represents the promoter/operator overlapping sequence. The RBS for rightward *(bioB)* transcription, GGAGAA, and the start codon for *bioA* and *bioB* genes are also marked. The dark boxes flanking the *bio* regulatory sequence are parts of the multiple cloning sites of vector pUC18. The abbreviations H, Sp, P, Sa, Xb, Xm, K, Sc, and E, represent *Hin*dIII, *Sph*I, *Pst*I, *Sal*I, *Xba*I, *Xma*I, *Kpn*I, *Sac*I, and *Eco*RI, respectively.

mid pUC11A. The resulting 216-bp *Eco*RI-*Hin*dIII fragment containing the *bio* operon regulatory sequence was then subcloned into M13mp18, and site-directed mutagenesis was performed *(21)*. The mutations are of three categories: insertion of a T between nt –18 and –19 to create a *Cla*I site (5'-ATCG AT-3') so that the RBS *Taq*I end can be ligated; a GC to TA mutation at nt –3, to create a better –10 region, 5'-TTAAAT-3', of a strong leftward promoter P_{98} *(19)*; and a GC to TA mutation at nt –3 as described

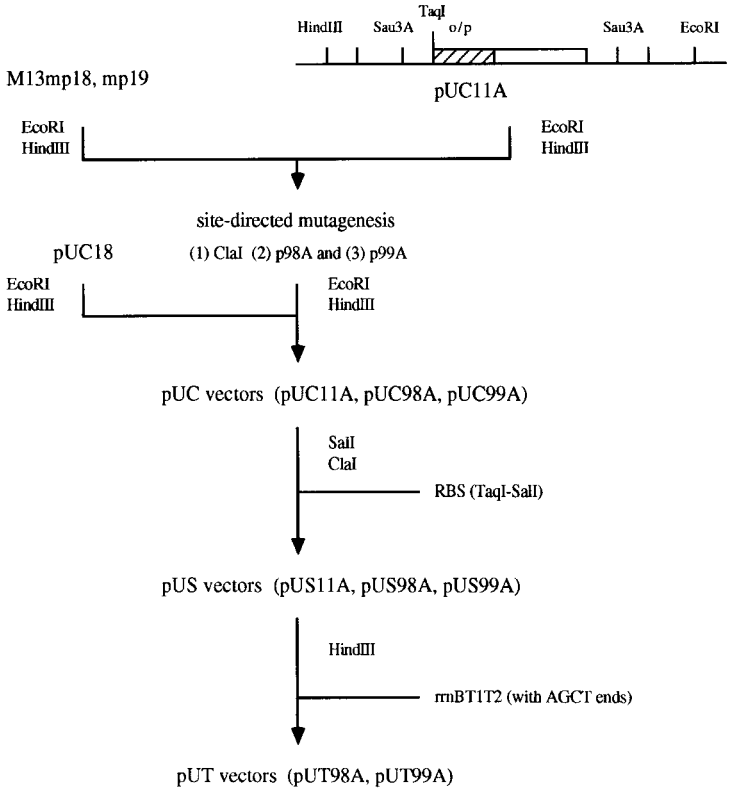

Fig. 2. The construction flow chart of pUC, pUS, and pUT series expression vectors. The 216-bp *Eco*RI-*Hin*dIII fragment was subcloned from plasmid pUC11A into M13mp18 for site-directed mutagenesis. The *Eco*RI-*Hin*dIII fragments of the resulting plasmids were then assembled into pUC18 to obtain the pUC vectors: pUC11A, pUC98A, and pUC99A. The synthesized RBS flanked with *Taq*I and *Sal*I sites was inserted into the *Cla*I-*Sal*I sites of pUC vectors to obtain the pUS vectors: pUS11A, pUS98A, and pUS99A, respectively. Finally, the pUS vectors were digested with *Hin*dIII and ligated with the synthesized rrnBT_1T_2 DNA fragment (with AGCT ends to clone into the *Hin*dIII site) to obtain the pUT vectors: pUT98A and pUT99A.

above, plus a TA to AT mutation at nt +21, to create a consensus –35 region, 5′-TTGACT-3′, a potentially stronger leftward promoter P_{99}.

The construction flow chart of the pUC, pUS, and pUT series expression vectors is outlined in Fig. 2. The 216-bp *Eco*RI-*Hin*dIII fragment was subcloned from plasmid pUC11A *(15)* into M13mp18 *(22)* and completed the site-directed mutagenesis, as mentioned. The *Eco*RI-*Hin*dIII fragments of the resulting plasmids were then assembled into pUC18 to obtain the pUC vectors: pUC11A, pUC98A, and pUC99A, respectively. The synthesized RBS, flanked with *Taq*I and *Sal*I sites (Fig. 3) was inserted into the *Cla*I-*Sal*I sites of pUC vectors to obtain the pUS vectors: pUS11A, pUS98A, and pUS99A, respectively. Furthermore, the pUS vectors were digested

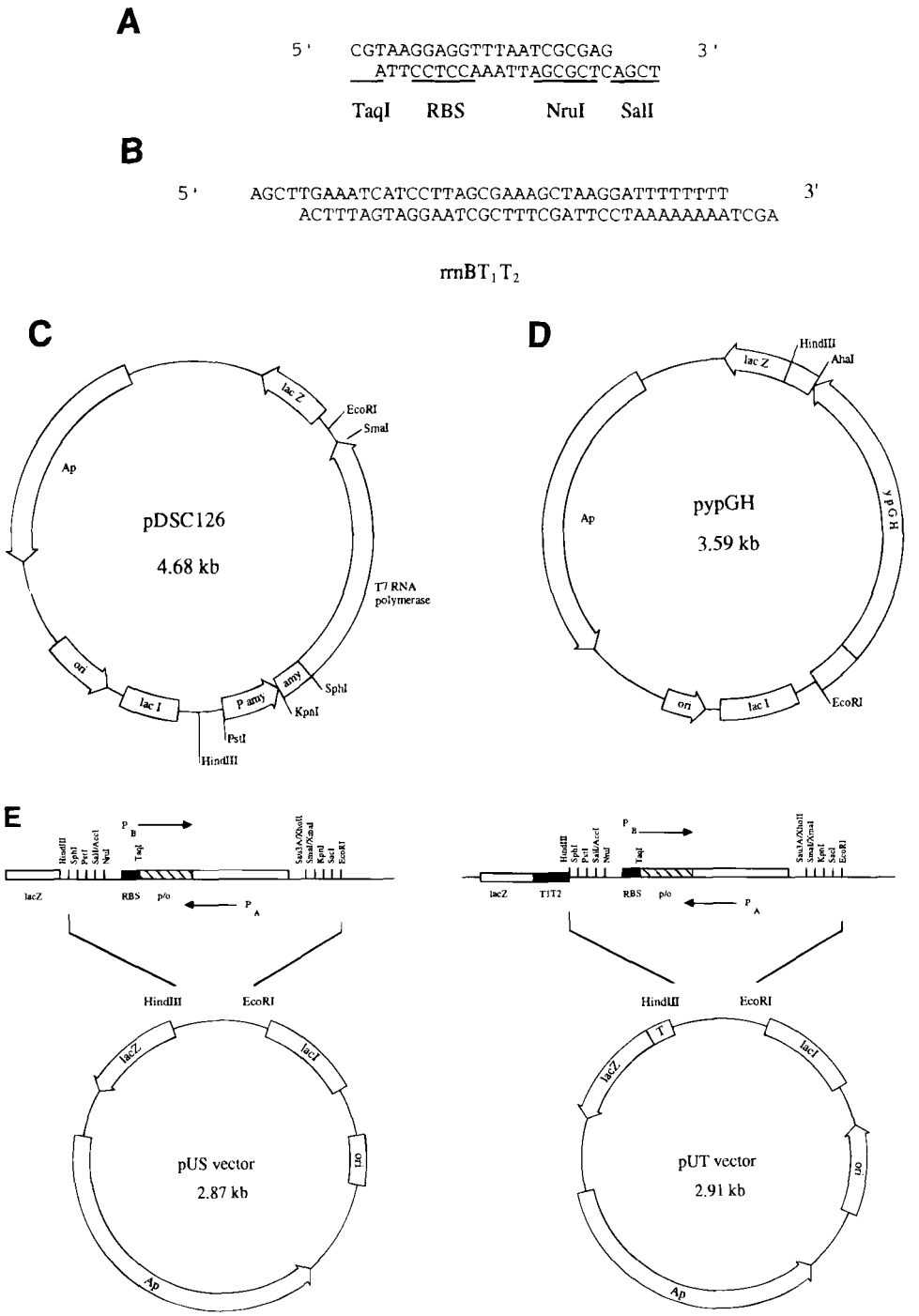

Fig. 3. Plasmids and DNA fragments used in constructing the expression vectors. **(A)** RBS, the ribosome-binding site or the Shine-Dalgarno sequence, AGGAGG, designed with a 5'*Taq*I site, a 3'*Sal*I site, and an *Nru*I site, 8 bp downstream from the RBS, was chemically synthesized and annealed. **(B)** The $rrnBT_1T_2$ fragment, a very strong transcriptional terminator, was designed with AGCT ends, compatible with

with *Hin*dIII and ligated with the synthesized rrnBT_1T_2 DNA fragment (with AGCT ends to insert into the *Hin*dIII sites) to obtain the pUT vectors: pUT98A and pUT99A, respectively.

Monitoring Biotin Concentration Change During Bacterial-Cell Growth

In evaluating the biotin-regulated gene expression system in *E. coli*, biotin of various concentrations was added to 1% T-broth, and the biotin concentration during host-cell growth was monitored using the competitive ELISA method *(23)* to establish the relationships between the onset of heterologous-gene expression and the deprivation of biotin during cell growth. As shown in Fig. 4, as the cell (strain R901 transformed with pUS11A-1, the pUS11A vector with *E. coli birA* gene cloned) entered the midlog phase (as OD_{600} increased from 0.1 to approx 1.0, described as open squares in Fig. 4), the medium biotin concentration (closed square in Fig. 4) dropped from 5 ng/mL to less than 2 ng/mL and triggered the overexpression of the inserted gene.

RESULTS AND DISCUSSION

The expression system was designed so that the induction of heterologous-gene expression could be triggered when biotin concentration dropped to a certain level and released the repression of the cloned gene caused by the binding of biotinyl-AMP (corepressor) and the biotin repressor to the biotin promoter/operator region *(18,19)*. The strain R901 with the *bio* operon deleted was unable to grow in the minimal medium *(24)* unless biotin was added. However, strain R901 grew well in 1% T-broth containing endogenous biotin of approx 6 ng/mL *(23)*. Therefore, the 1% T-broth was chosen as the basal medium to optimize the gene-expression system. The marker genes encording: *E. coli* biotin repressor; T7 RNA polymerase; and yellowfin-porgy growth hormone, were used to test the feasibility of the expression system. These genes were cloned into vectors

Fig. 3. *(continued)* *Hin*dIII site for cloning. The fragments were also chemically synthesized and annealed before cloning. **(C)** Plasmid pDSC126, this plasmid was constructed by one of us (D. S.) for preparing a T7 RNA polymerase-regulated gene expression system in *Streptomyces lividans* TK24. The *Sph*I-*Sma*I fragment containing the 1.2-kb T7 RNA polymerase gene, was inserted downstream of the α-amylase promoter and the codons of the first ten amino acid residues of the α-amylase gene of *Streptomyces lividans* TK24 were replaced with *E. coli*-preferred codons. **(D)** Plasmid pypGH containing the growth hormone gene of yellowfin porgy *(18)*, was a generous gift from H. J. Tsai, Institute of Fisheries Science, National Taiwan University. **(E)** Plasmid vector pUS and pUT series, these vectors were constructed by assembling suitable regulatory regions including the biotin operator/promoter overlapping region, the RBS and the transcription terminator in the multiple cloning sites of plasmid pUC18, as described in Fig. 2.

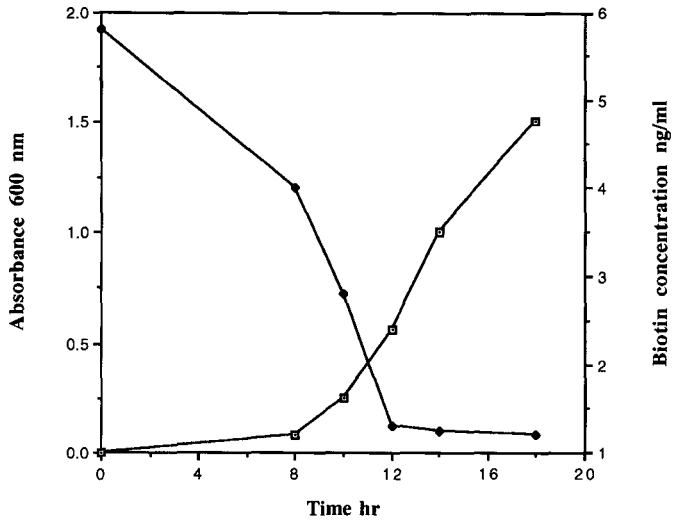

Fig. 4. Depletion of biotin during cell growth. Overnight culture of *E. coli* strain R901 harboring the expression plasmid was diluted 1:100 in 1% T-broth (per liter: 5 g NaCl, 10 g tryptone, 0.1 g thiamine HCl, with endogenous biotin of approx 6 ng/mL) and shaken at 220 RPM at 37°C overnight. Samples were collected every 2 h for determination of biotin concentration using the modified competitive ELISA method (23). The cell density was estimated by measuring OD_{600}. The closed and open squares represent the changes of biotin concentrations and cell density, respectively.

pUS11A, pUS98A, and pUT98A, to obtain plasmids pUS11A-1, pUS98A-1, pUT98A-1, pUS11A-2, pUS98A-2, pUS99A-2, pUS98A-3, and pUT98A-3, as indicated in Table 1. The expression patterns of these constructs were examined separately.

We first transformed R901 with pUS11A-1 and grew it in 1% T-broth at 37°C, 220 RPM, to observe the onset of overexpression of the cloned *birA* gene. The gene expression patterns can be visualized by SDS-polyacrylamide gel electrophoresis of the total *E. coli* protein at various growth stages. As shown in Fig. 5A, as the OD_{600} reached above 0.58 (lane A4, corresponding to biotin concentration of approx 1 ng/mL from Fig. 4), the 35 kDa protein started to accumulate abruptly and high-level expression (approx 25% of total protein by laser densitometry) was continued until OD_{600} = 1.54. As extra biotin 0.1 μg/mL (Fig. 5B) was added initially into the 1% T-broth, the onset of overexpression was found to be delayed to approximately OD_{600} = 1.44 (lane B5). Similar expression patterns were also observed for the yellowfin-porgy growth-hormone gene on plasmid pUT98A-3. As shown in Fig. 6, the expression of the 28.5 kDa growth-hormone gene can be turned on as the biotin concentration dropped to approx 1 ng/mL. The effects of varied promoter strength on the expression pattern were basically as expected. The yellowfin-porgy growth-hormone gene was inserted into vectors of increasing promoter

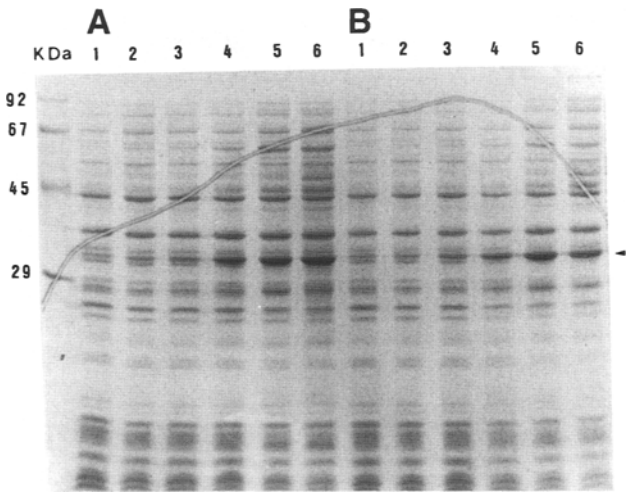

Fig. 5. Expression of *E. coli birA* gene in 1% T-broth medium. The 0.95-kb *E. coli birA* gene was synthesized by PCR method (*26*). The primers are (I) 5′-ATGAAG-GATAACACCGTG-3′, the 5′ end of the *birA* gene, and (II) 5′-ATATAAGCT-TAGCGCGCCAGTTTAATCC-3′, complements the DNA sequence approx 60 bases downstream from the *birA* terminator, with a designed *Hin*dIII site at the distal end for cloning (*17*). The 0.95-kb DNA fragment was gel purified after PCR reaction, digested with *Hin*dIII and cloned into the plasmid vector pUS11A to obtain pUS11A-1. **(A)** *E. coli* strain R901 harboring pUS11A-1 was grown in 1% T-broth at 37°C and samples were collected at OD_{600} = 0.06, 0.18, 0.36, 0.58, 1.10, and 1.54. The total protein of each sample was analyzed by 12% SDS polyacrylamide gel electrophoresis. The 35 kDa protein, the *birA* gene product of each sample, represents 2.0, 5.3, 12.6, 17.0, 21.0, and 23.0% of the total *E. coli* cellular protein, respectively, by laser densitometry (with a laser densitometer LKB22220-020, Ultra Scan XL, data not shown). **(B)** Extra biotin (0.1 μg/mL) was added initially into the 1% T-broth to observe the effect on induction of gene expression. The transformed cell was grown at 37°C until OD_{600} = 0.09, 0.25, 0.45, 0.82, 1.44, and 1.86, and the total proteins analyzed similarly.

strength and obtained pUS11A-3, pUT98A-3, and pUT99A-3, respectively. Under the same experimental conditions, the induction patterns of the marker gene controlled by promoters of varied strength were very similar, but the amounts of the growth hormone were found to be approximately threefold higher using plasmid pUT98A-3 than plasmid pUS11A-3 (data not shown). However, the expression level using plasmid pUT99A-3, with a potentially stronger promoter was not significantly different from that of pUT98A-3 (data not shown).

The overexpression of T7 RNA polymerase gene could be detrimental to *E. coli (6)* because the growth of R901 carrying the plasmid pUS11A-2 was impaired as medium biotin level was reduced. The effect of adding 2 μ*M* avidin into the medium in order to neutralize the biotin concentration (by forming biotin-avidin complex), thus possibly inducing the gene expression, however, was not obvious (data not shown), probably due to

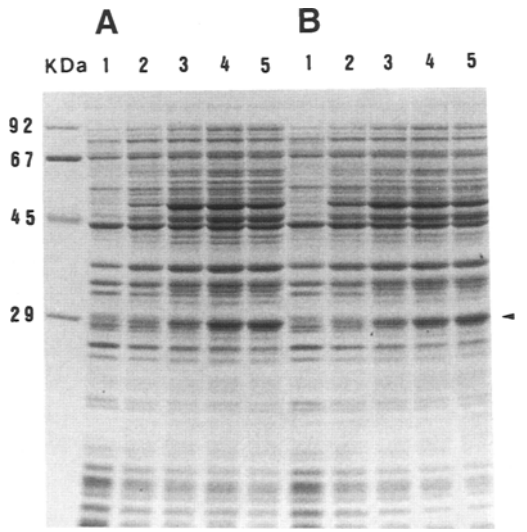

Fig. 6. Expression of the yellowfin porgy growth hormone (ypGH) gene. The ypGH gene was subcloned from plasmid pypGH by PCR. The designed primers are (I) 5′-ATGCAGCCGATCACCGACGG-3′, containing the initiation codon ATG added to the 5′ end of the ypGH gene. The codons of the first five amino acid residues of ypGH gene were changed to meet the codon preference of *E. coli*. (II) 5′-GTAAAACGACG-GCCAGT-3′, complements the right edge of the multiple cloning sites of plasmid pypGH. The 0.87-kb PCR product was cleaved with *Hin*dIII (located just downstream of the transcription terminator of ypGH gene) and ligated to the expression vector pUT98A (digested with *Nru*I and *Hin*dIII) to obtain plasmid pUT98A-3. *E. coli* strain R901 was transformed with pUT98A-3 and grown in **(A)** 1% T-broth until OD_{600} = 0.08, 0.36, 0.80, 1.13, and 1.60, and the total proteins analyzed by 12% SDS polyacrylamide gel electrophoresis, **(B)** 1% T-broth plus biotin 100 ng/mL. Samples were analyzed by 12% SDS polyacrylamide gel electrophoresis at OD_{600} = 0.09, 0.38, 0.83, 1.22, and 1.66.

its limited permeability across the cell membrane. In the current system, the expression of cloned genes can be triggered simply by the depletion of medium biotin during cell growth, providing many advantages over other systems induced by heat shock or chemical inducers. In addition, the corepressor (biotin) depletion does not interfere with transcription or translation of other genes, but rather blocks fatty-acid synthesis *(25)*. Blocking fatty-acid synthesis is bacteriostatic and metabolically rather benign, because no cell lysis results and other metabolic pathways proceed. However, the comparatively poor growth medium (the 1% T-broth) has to be improved in order to reach high-cell densities in a fermentor and produce high concentrations of recombinant proteins.

In conclusion, the results demonstrated that the induction of marker-gene expressions could be triggered as the biotin concentration dropped to a threshold value of approx 1 ng/mL by the metabolic utilization during cell growth. The 1% T-broth medium seems to be appropriate for the gene-expression system. The expression levels of the cloned gene can reach as

high as 25% of the total cellular protein, comparable to the levels of heat-induced *(17)* and IPTG-induced systems. The gene-expression system was shown to be regulated simply by the decrease in biotin concentration, overcoming many disadvantages such as heat-induced proteolysis and difficult final removal of the added chemical inducer in large-scale fermentation associated with the currently used systems regulated by heat shock or by added chemical inducers. Although the control pattern of this system does not constitute a very tight on–off switch, it should be applicable to the overexpression of foreign-gene products, which are not extremely toxic to the cells.

ACKNOWLEDGMENT

The research was supported by grants NSC81-0211-B110-508 and NSC83-0211-B110-001 from the National Science Council, Republic of China. We thank Steven Li, NIEHS (Research Triangle Park, NC) and Pearl Lin of the Development Center for Biotechnology (Taipei, Taiwan) for reading the manuscript and for many valuable suggestions.

REFERENCES

1. Itakura, K., Itirose, T., Crea, R, and Riggs, A. D. (1977), *Science* **128,** 1056–1063.
2. Miyanohra, A., Tohe, A., Nozaki, C., Hamada, F., Ohtome, N., and Matsubara, K. (1983), *Proc. Natl. Acad. Sci. USA* **80,** 1–5.
3. Mori, T., Yamamoto, K., Ohta, T., Sakamoto, C., Sato, M., Koide, K., Murakami, T., Fujii, M., Fulcuda, S., and Kurimoto, M. (1984), *Gene* **144,** 289–293.
4. Mylin, L. M., Hofman, K. J., Schultz, L. D., and Hopper, J. E. (1978) in *Methods in Enzymology*, vol. 185, Goeddel, D. V., ed., Academic Press, NY, pp 297–308.
5. Desutter, K., Hostens, K., Vandekerckhove, J., and Fiers, W. (1994), *Gene* **141,** 163–170.
6. Studier, W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1990) in *Methods in Enzymology*, vol. 185, Goeddel, D. V., ed., Academic Press, NY, pp. 60–89.
7. George, H. J., Watson, R. J., Harbrecht, D. F., and Delorbe, W. J. (1987), *Bio/Technology* **5,** 600–603.
8. Chen, X., Li, Y., Xiong, K., and Wagner, T. E. (1994), *Nucleic Acid Res.* **22,** 2114–2120.
9. Duport, C., Meyer, C., Naud, I., and Jouanneau, Y. (1994), *Gene* **145,** 103–108.
10. Etcheverry, T. (1990) in *Methods in Enzymology*, vol. 185, Goeddel, D. V., ed., Academic Press, NY, pp. 319–329.
11. Roder, F. T., Schmulling, T., and Gatz, C. (1994), *Mol. Genet.* **243,** 32–38.
12. Su, T. Z., Schweizer, H., and Oxender, D. L. (1990), *Gene* **90,** 129–133.
13. Cleary, P. P. and Campbell, A. (1972), *J. Bacteriol.* **112,** 830–839.
14. Barker, D. F., Kuhn, J., and Campbell, A. (1981), *Gene* **13,** 89–102.
15. Shiuan, D. and Campbell, A. (1988), *Gene* **67,** 203–211.
16. Gentz, R., Langner, A., Chang, A. C. Y., Cohen, S. N., and Bujard, H. (1981), *Proc. Natl. Acad. Sci. USA* **78,** 4936–4940.
17. Lin, K. C., Campbell, A., and Shiuan, D. (1991), *Biochim. Biophys. Acta.* **1090,** 317–325.
18. Tsai, H. J., Liu, K. L., and Chen, T. T. (1993), *Comp. Biochem. and Physiol.* **104B,** 803–810.
19. Otsuka, A. and Abelson, J. (1978), *Nature* **276,** 689–694.
20. Shiuan, D., Lin, K. C., and Campbell, A. (1994), *Gene* **145,** 1–7.

21. Kunkel, T. (1985), *Proc. Natl. Acad. Sci. USA* **82,** 488–492.
22. Yanisch-Perron, C., Vieira, J., and Messing, J. (1985), *Gene* **33,** 103–119.
23. Chang, Y. S., Wu, C. H., Chang, R. J., and Shiuan, D. (1994), *J. Biochem. Biophys. Methods* **29,** 321–329.
24. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982), *Molecular Cloning: A Laboratory Manual* pp. 68, 69, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
25. Cronan, J. E. (1989), *Cell* **58,** 427–429.
26. Saiki, R. K., Gelfand, D. H., Stoffel, S., Schare, S. I., Higuchi, R., Horn, G. T., Mullis, K. B., and Erlich, H. A. (1987), *Science* **239,** 489–494.
27. Hanahan, D. (1983), *J. Mol. Biol.* **166,** 557–580.
28. Vieira, J. and Messing, J. (1982), *Gene* **19,** 255–268.