Vol. 170, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1990 Pages 1-9

TEMPERATURE DEPENDENCE OF TRANSCRIPTIONAL ACTIVITY OF YEAST 3-PHOSPHOGLYCEROKINASE PROMOTER IN ESCHERICHIA COLI

Alok K. Mondal and Tamal K. Roy*

Institute of Microbial Technology
Post Box No. 1304, Chandigarh 160026, INDIA

Received May 15, 1990

SUMMARY: We have studied the activity of yeast PGK promoter in E. coli at different temperatures using lacZ as the reporter gene. The B-galactosidase activity was found to be less at 42°C than at 30°C. Northern blot analysis showed that the level of lacZ transcript was less in recombinant cells grown at 42°C, whereas plasmid copy number per cell was more as compared to recombinant cells grown at 30°C. Data suggest that the yeast PGK promoter is less active at 42°C and that this activity is regulated at the level of transcription. © 1990 Academic Press, Inc.

The promoter of 3-phosphoglycerokinase (PGK) gene of yeast, Saccharomyces cerevisiae, is one of the strong constitutive promoters (1). This gene was first cloned and identified by immunological screening in Escherichia coli (2). The PGK promoter of S. cerevisiae has been used to express a variety of cloned genes in yeast (2-6). Besides, several yeast genes have also been used with their own promoter to complement appropriate mutation in E. coli (7). However, no attempt has been made to test the activity of PGK promoter and its suitability for expression of genes in E. coli. In order to fill up this lacuna we undertook to determine the activity of yeast PGK promoter in E. coli at different temperatures. This communication reports that the activity of yeast PGK promoter in E. coli is temperature dependent.

MATERIALS AND METHODS

Strains and plasmids: E. coli strain TB1 (lac Z Δ M15, r^-,m^+, ara, rspl, O 80, proAB, Sm^R), was obtained from Dr. B.J. Johnson, CDC, Fort Collins, USA, UT580 (r^-, m^+, sup D, pro lac Δ , p' Tn10) was obtained from Dr. R.D. Pridmore, Nestec Ltd., Switzerland as BZ234 and K802 ($hsdR^+$, $hsdM^+$, gal^-, met^-, supE) was obtained

* Deceased.

0006-291X/90 $1.50
Copyright © 1990 by Academic Press, Inc.
All rights of reproduction in any form reserved.

from Dr.P.K. Maitra, Tata Institute of Fundamental Research, Bombay, India. Plasmid pMBL216 (8) was obtained from Dr. B.E. Enger-Valk, Medical Biology Laboratory TNO, The Netherlands. S. cerevisiae strain W303-1B (α -leu2-3, 112, his 3-11, 15 ade 2-1, ura 3-1 trp 1-1. cau 1-100 [cir$^+$] was from Dr. D.M. Kinney, Public Health Research Inst., New York.

Nucleic acids manipulations and transformation: Plasmid DNA was isolated by method of Birnboim and Doly (9). Restriction enzymes and other DNA modifying enzymes were used as recommended by suppliers. Total RNA from E. coli cells was isolated as described by Frederick et al. (10). Plasmid copy number was determined by method of Korpela et al.(11). Transformation of E. coli strains was done by method of Mandel and Higa (12). Total RNA from yeast was isolated as described by Feinberg et al (13).

Northern blot: Gel electrophoresis of RNA and northern blot was done as described by Frederick et al. (10). The lac Z gene purified from pMBL216 was used as the hybridization probe. Nick translation was done(14) using α [^{32}P]-dCTP or α [^{32}P]-dGTP (Bhabha Atomic Research Centre,Bombay, India).

Assay of β-galactosidase: The β-galactosidase activity in cell homogenates was measured at 420 nm using O-nitrophenyl-β-D-galacto-pyaranoside (ONPG) as substrate (15). Briefly, cells were grown in 10ml LB medium to logarithmic phase, harvested at 5K rpm for 5 minutes, washed once with B-gal buffer (Tris-HCl, pH 7.5, 50mM; KCl, 10mM; $MgSO_4$, 1mM; 2-ME, 50mM), and suspended in 2.5 ml B-gal buffer. Cells were broken with glass beads in MSK homogenizer (Braun, FRG). Homogenates were centrifuged at 10K rpm at 2°C for 10 minutes. Supernatants were used for enzyme assay with 1mM ONPG. Protein was estimated by method of Lowry et al (16).

Construction of vector: Plasmid pMBLf1 was constructed by digesting pMBL216 with SmaI and Bal31. In pMBL216 the lac Z gene is out of ATG frame (figure 1A). Therefore, pMBLf1 was selected by plating E. coli TB1 cells, transformed with SmaI and Bal31 digested and blunt end ligated pMBL216 DNA on plates containing 5-bromo-4-chloro-3-indolyl-B-D-galactopyranoside (X-gal). A blue colony was picked and plasmid DNA was prepared from that clone. Plasmid pMBLf1 was digested with several enzymes to determine the extent of Bal31 digestion of pMBL216 (figure 1B).

Primer extension analysis (17): A 3.2 kb Bam H1 fragment from plasmid pMBLf1 was labeled by E. coli DNA polymerase 1 (Klenow fragment) using α [^{32}P]-dGTP and was subsequently subjected to Sau 3A digestion to yeild a 96 bp fragment labelled in the anti mRNA strand. The 96 nucleotide primer was used for hybridization with total RNA isolated from yeast and E. coli. Yeast t-RNA was added when necessary to make a total of 125 ug of nucleic acid in all reactions. Hybridizations were carried out with probe excess for 16h at 57°C in 50 ul of formamide hybridization buffer (80% formamide, 0.4M NaCl, 10mM pipes pH 6.4, 1mM EDTA). Reactions were quenched with 3M sod. acetate and precipitated with ethanol. Samples were dried and dissolved in 30 ul of reverse transcriptase buffer. 21U of AMV reverse transcriptase was added. After 1 hr. incubation at 42°C, reaction mixture was treated with RNAse, extracted with phenol, precipitated with ethanol and then analysed on a 6% sequencing gel.

S1 Nuclease protection assay: S1 mapping was carried out by the method of Berk and Sharp (18) A 2.0 kb Bam H1 Pst I fragment

was dephosphorylated and labeled at the 5' end by T4 polynucleotide Kinase and [γ^{32}P] ATP (3000Ci/mmol). It was digested with Rsa1 to generate a 247 nucleotide probe. Total RNA was hybridized to end-labeled probe DNA for 16h, at 57°C in 50ul formamide hybridization buffer. Samples were alcohol precipitated, dried, dissolved in 100ul of S1 nuclease buffer. (30mM Na-acetate pH 4.6, 100 mM NaCl, 1mM Zn SO_4, 200 ug calf thymus DNA/ml) and incubated with 100 U of S1 nuclease at 37°C. After 1h of incubation samples were extracted with phenol, ethanol precipitated and analysed on a 6% denaturing polyacrylamide gel.

RESULTS AND DISCUSSION

Appearance of blue colonies of E. coli cells containing pMBLf1 on X-gal plates showed that the lac Z gene was being expressed through yeast PGK promoter. When cells containing pMBLf1 were streaked on X-gal plates (20ug/ml) and incubated at 30°C, 37°C and 42°C, the colonies were blue only at 30°C and 37°C. However, with a higher concentration of X-gal (40ug/ml) in plates, colonies were blue also at 42°C (data not shown), indicating that cells grown at 42°C do synthesize β-galactosidase though at a very low level. Since normal temperature of growth of S. cerevisiae is lower than that of E. coli, an attempt was made to determine the quantitative level of PGK promoter activity at three different temperatures in E. coli.

As evident from table 1, the activity of β-galactosidase in pMBLf1 was maximum at 30°C, which was 1.3 times more than that at 37°C and five times more than that at 42°C. In order to prove that observed difference in enzyme activity was not strain dependent, we used another plasmid, pMBLf10R1 (map not shown) which is a derivative of pMBLf1 in a different E. coli host, UT580. The enzyme activity in pMBLf10R1 at 30°C was two times and 3.5 times more than at 37°C and 42°C, respectively (table 1). The basal level of β-galactosidase activity in TB1 and UT580 was not detectable (data not shown).

To determine whether cells can produce enzymatically active β galactosidase at higher temperature, we grew E. coli K802 which carries a functional lac operon at 30°C, 37°C and 42°C. No significant difference in enzyme activity could be observed in cells grown at these temperatures (table 1). The level of enzyme activity in IPTG (isopropyl-β-D-thiogalactopyranoside) induced (10mM) K802 was 100 to 600 fold more than those in TB1/ pMBLf1 or UT580/pMBLf10R1. This was because the expression

Table 1: Beta-galactosidase activity in E.coli strains expressed through yeast PGK promoter

E.coli Strain	Plasmid	Growth Temperature	Enzyme Activity (nmole ONPG degraded per minute per mg protein)
TB1	pMBLf1	30°	2.56 ± 0.44
		37°C	1.92 ± 0.43
		42°C	0.54 ± 0.12
UT580	pMBLf1OR1	30°C	2.69 ± 0.51
		37°C	1.3 ±0.64
		42°C	0.78 ± 0.42
K802	-	30°C	326.89 ± 34.62
		37°C	314.00 ± 20.58
		42°C	372.40 ± 17.38

of lac Z gene in K802 was under the lac promoter control. Low level of enzyme activity in E. coli carrying pMBLf1 could be either because of the lac Z gene being under the control of yeast promoter in a heterologous system or due to the nucleotides removed by Bal31 digestion in cloning step. Although the number of bases removed by Bal 31 digestion was not accurately determined by sequencing, an analysis of the restriction pattern revealed that (figure 1B) the number of bases that might have been removed at the 5' promimal end of the Sma I site of pMBL216 was between 24 and 30. Assuming that the same number of bases have been removed towards 3' of SmaI site, a minimum 15 and a maximum 21 bases might aslo have been removed from 5' end of lac Z gene. Therefore, total number of amino acid codons of lac Z gene removed in pMBLf1 from its 5' end would be between 13 and 15 (including 8 amino acid codons already removed in pMBL216) (8).

Since the removal of 27 amino acid codons from 5'end of lac Z gene is known not to affect the β-galactosidase activity (19) it is apparent that most probably the expression of lac Z gene in pMBLf1 is regulated at the level of transcription. To verify this further we did northern blot analysis of cells grown at 30°C and 42°C (figure 2). It can be seen from figure 2 that the amount of transcript of lac Z gene is more at 30°C than that at 42°C. To further confirm that the same promoter being utilized both in E. coli and in yeast a Primer extension and S1 nuclease protection analysis was done with total RNA isolated from yeast

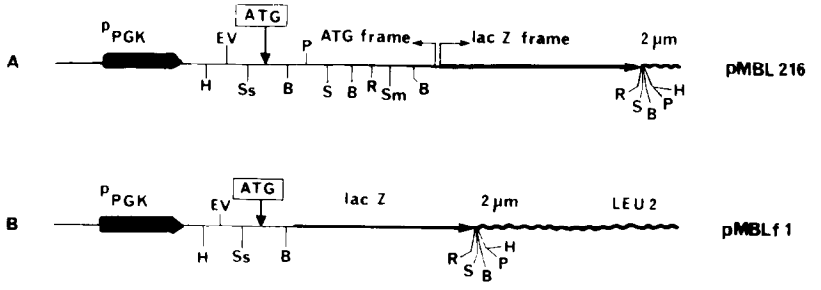

Fig.1. Linear map of pMBL216 and pMBLf1. (A) in pMBL216 the lac Z gene is out of ATG frame by one base. (B) In pMBLf1 the lac Z gene has been brought in-frame of ATG (see text). Deletion of restriction sites in pMBLf1 was determined by digestion with EcoRI, SalI, BamHI and PstI. B = BamHI, EV = EcorV, H = Hind III, P = Pst I, R = EcoRI, S = Sal I, Sm = SmaI, Ss = SstI. Not drawn to scale.

grown at 30°C and E. coli grown at 30°C and 42°C. The length of extended product (fig.3) and the length of the protected fragment (fig.4) was found to be the same in yeast and E. coli, indicating that the same promoter was being utilized in both the organisms. The low intensity of bands in lane 2 (fig3) and lane 3 (fig.4), in spite of the fact that five times more RNA was used for hybridization, was also indicative of less amount of transcripts being

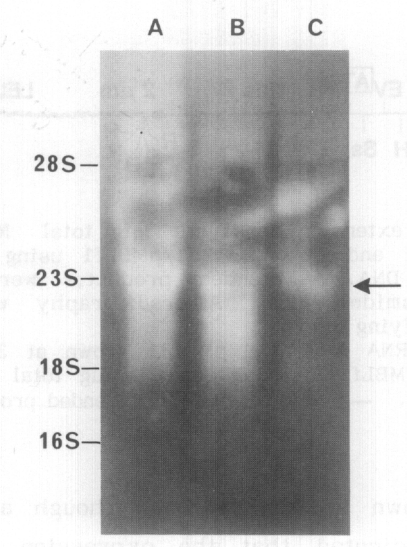

Fig. 2. Northern blot of total RNA isolated from cells. Lane A, pMBLf1 grown at 30°C; lane B, pMBLf1 grown at 42°C; lane C, pUC18 as control. 20 ug of total RNA of each sample was loaded and run on 1.2% formaldehyde - agarose gel. RNA was transferred to zeta probe blotting membrane (Bio Rad, USA) for hybridization (see text). Arrow indicates the position of transcript of lac Z gene. Molecular weight markers are on the left side of the figure.

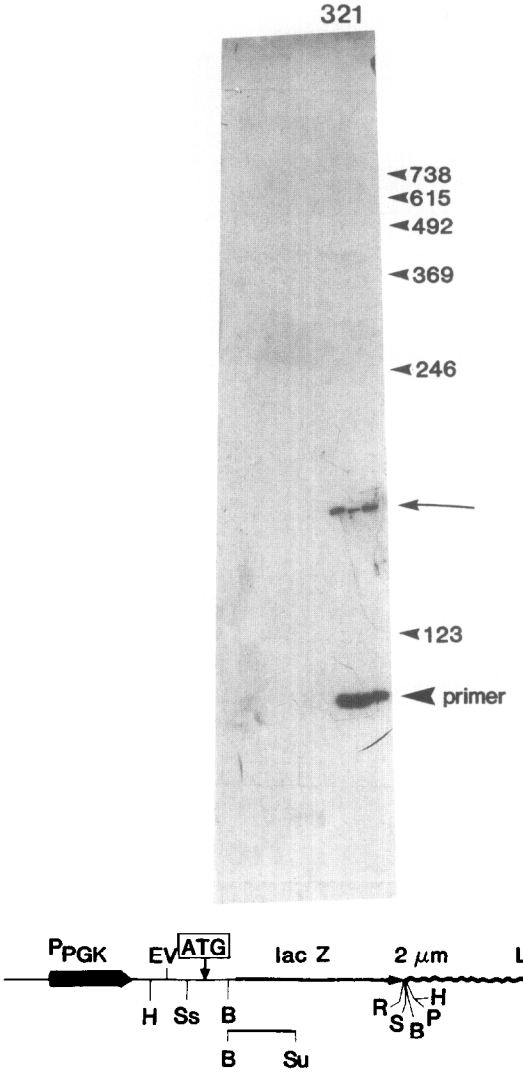

Fig. 3. Primer extension analysis of total RNA isolated from yeast W303-1B/pMBLf1 and E. coli TB1/pMBLf1 using the 96 nucleotide BamH1-San3A primer DNA. Extended product,s were analyzed on 6% denaturing polyacrylamide gel. Autoradiography was for 36hr. at -70°C with an intensifying screen.
Lane-1, 25 ug total RNA from TB1/pMBLf1 grown at 30°C Lane 2. 125ug total RNA from TB1/pMBLf1 at 42°C Lane 3-25ug total RNA from W303-IB/pMBLf1 grown at 30°C. ⟶ indicates the extended product.

made in E. coli grown at 42°C. Even though all the experiments described so far indicated that the expression of Lac Z gene in pMBLf1 is regulated at the level of transcription, it could still be argued that the observed difference in the level of lac Z transcripts at two temperature was simply a gene dosage effect arising out of a difference in the number of copies of pMBLf1 present in the cells at these two temperature. A determination

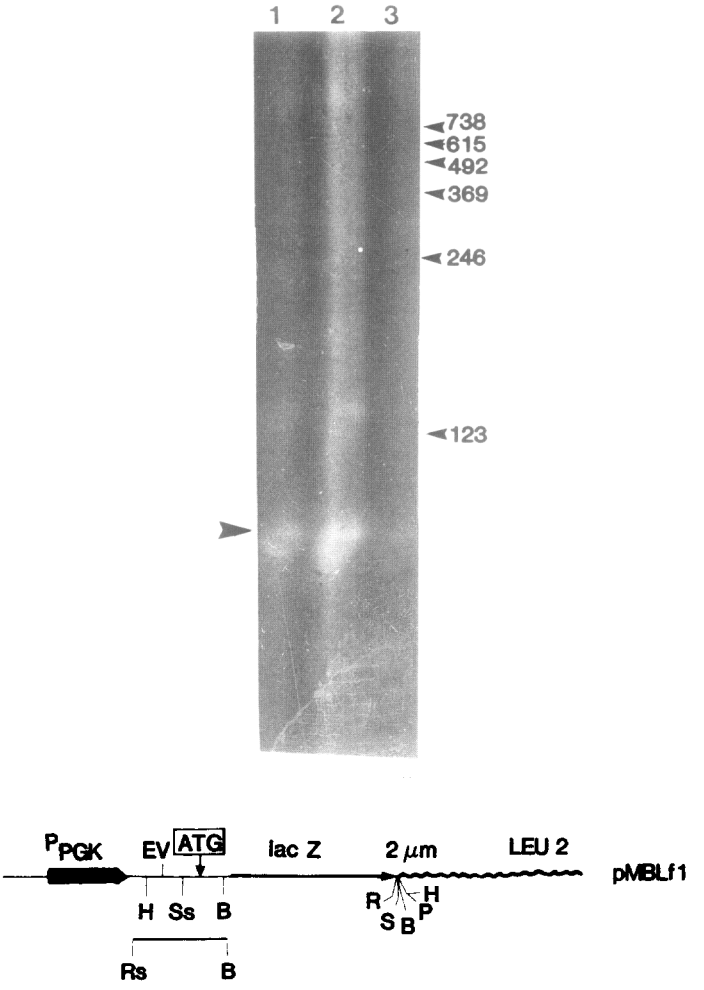

Fig. 4. S1 nuclease protection analysis of total RNA isolated from W303-1B/pMBLf1 and TB1/pMBLf1 using 247-nucleotide RsaI-Bam H1 probe. RNA was hybridized for 16h at 57°C to end-labeled probe and hybrids were treated with S1 Nuclease (Amersham) and digestion products were analysed on 6% denaturing polycerylamide gel. Autoradiography was for 3 days at - 70°C with intensifying screen. Lane 1. total RNA from W303-1B/pMBLf1 grown at 30°C Lane-2 25ug of total RNA from TB1/pMBLf1 grown at 30°C, Lane 3-125ug of total RNA from TB1/pMBLf1 grown at 42°C. ➤ indicates the protected fragment.

of plasmid copy number in cells grown at 30°C and 42°C showed that plasmid copy number per cell was more at 42°C than at 30°C (table 2). This not only ruled out the possibility alluded to above but also provided a clear indication that yeast PGK promoter is less active at 42°C. Earlier studies (20) have shown that heat shock sometimes increased the activity of wild type PGK promoter in yeast and that heat induction of promoter activity was pronounced when the upstream activation sequence (UAS pGK) in the promoter was deleted. Thus it appears that the heat induction

Table 2: Copy number of pMBLf1 in E.coli TB1 grown at different temperatures

Growth temperature	Plasmid copy number per cell
30°C	154.45 ± 5.5
42°C	227.89 ± 28.6

has opposite effects in the transcriptional activity of pGK promoter depending upon wehter it is in yeast or in E. coli.

Yeast PGK promoter is not the only yeast promoter which has been shown to be active in E. coli. There are other yeast genes like URA3, TRP1, LEU2 which have been used with their own promoter to complement a corresponding mutation in E. coli (7). To the best of our knowledge, however, this the first demonstration of the differential activity of a yeast promoter in E. coli. We have also observed similar phenomenon in northern blot analysis of yeast URA3 and TRP1 transcripts in E. coli (data not shown). Work is in progress to understand the mechanism of the observed temperature dependent activity of this promoter.

ACKNOWLEDGMENTS: We are thankful to Dr. Amit Ghosh and Dr. G.C. Mishra for advice and comments on the manuscript and Dr. S.K. Basu for helpful discussion. The expert technical assistance of Mr. Parmil Bansal, Mr. Raj Kumar and Ms. Nisha Dogra is gratefully acknowledged.

REFERENCES

1. 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, 603-608.
2. Hitzeman, R.A., Clarke, L. and Carbon, J. (1980) J. Biol. Chem. 255, 12073-12080.
3. Belsham, G.J., Barker, D.G. and Smith, A.E. (1986) Eur. J. Biochem. 156, 413-422.
4. Bodmer, M.W., Angal, S., Yarranton, G.T., Harris, T.J.R., Lyons, A., King, D.J., Pieroni, G., Riviera, C., Verger, R. and Lowe, P.A. (1987) Biochim. Biophys. Acta 909, 237-244.
5. Briton, P., Carmenes, R.S., Page, K.W., Garwes, D.J. and Parra, F. (1988) Mol. Microbiol. 2, 89-100.
6. Cullin, C. and Pompon, D. (1988) Gene 65, 203-218.
7. Sikorski, R.S. and Hieter, P. (1989) Genetics 122, 19-27.

8. Verbakel, J.M.A., Dekker, K.A., Rutgers, C.A., Pouwels, P.H. and Enger-Valk, B.E. (1987) Gene 61, 207-215.
9. Birubiom, H.C. and Doly, J. (1979) Nucl. Acids Res. 7, 1513-1523.
10. Frederick, M.A., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1989) Current Protocols in Molecular Biology, Vol. I. John Wiley and Sons, New York.
11. Korpela, K., Buchert, P. and Soderlund, H. (1987) J. Biotech. 5, 267-277.
12. Mandel, M. and Higa, A. (1970) J. Mol. Biol. 53, 159-162.
13. Feinberg, B. and Melanghlin, C.S. in "Yeast - a practical approach" eds compbel I and Duffus J.H., IRL Press, Oxford. pp-157.
14. Rigby, J.B., Dieckerman, M., Rhodes, C. and Berg. P. (1977) J. Mol. Biol. 113, 237-251.
15. Miller, J.H. (1972) Experiments in Molecular Genetics. pp. 352-359. Cold Spring Harbor Laboratory, Cold Spring, New York.
16. Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
17. Current Protocols in Molecular Biology (Ausubel, F.M. eds), John Wiley & SOns, New York, pp. 4.6.1-4.6.10.
18. Ber, A.J. and Sharp, P.A. (1977) Cell 12, 721-732.
19. Casadaban, M.J., Martinez-Arias, A., Shapira, S.K. and Chou, J. (1983) Meth. Enzymol. 100, 293-308.
20. Piper, P.W. et al (1988) Nucleic Acids Research 16, 1333-1348.