```
pSBCl
  Ser Leu His Ala Cys Ser Thr Thr Leu Glu Asn Pro Arg Val Pro Ser Ser
5 AGC UUG CAU GCC UGC UCG ACG ACA CUC GAG AAU CCU CGA GUG CCG UCG AGC 3
   5       7       9       11      13      15      17      19      21

pSBmCl
5 AGC UUG CAU GCC UGC AGC ACA ACG CUC GAG AAU CCG CGA GUG CCU UCG UCG 3
   5       7       9       11      13      15      17      19      21
```

The sequence synthesized and used to construct pSBmCl by replacing the *EcoRI HindIII* fragment of pUC19

```
5 AGC TTG CAT GCC TGC AGC ACA ACG CTC GAG AAT CCG CGA GTG CCT TCG TCG-3
           7       9       11      13      15      17      19
      3 AC GTA CGG ACG TCG TGT TGC GAG CTC TTA GGC GCT CAC GGA AGC AGC TTA A 5
```


1. Darnell, J. E., *Nature*, 1982, **297**, 365.
2. Struhl, K., *Annu. Rev. Biochem.*, 1989, **58**, 1051.
3. Proudfoot, N. J, *Trends Biochem. Sci*, 1989, **14**, 105.
4. Bagga, R., Ramesh, N. and Brahmachari, S. K., *Nucleic Acids Res.*, 1990, **18**, 3363.
5. Bentley, D L. and Groudine, M, *Cell*, 1988, **52**, 245.
6. Mok, M., Maderious, A. and Chen-Kiang, S., *Mol. Cell. Biol.*, 1984, **4**, 2031.
7. Peck, L. J. and Wang, J. C., *Cell*, 1985, **40**, 129
8. Kinniburgh, A. J., *Nucleic Acids Res.*, 1989, **17**, 7771.
9. Horowitz, M S. and Loeb, L. A, *Science*, 1988, **241**, 703.
10. Brahmachari, S. K. *et al.*, in *Structures and Methods* (eds Sarma, R H. and Sarma, M. H), Adenine Press, New York, 1990, vol. 2, p. 33.
11. Brahmachari, S. K., Mishra, R. K., Bagga, R. and Ramesh, N., *Nucleic Acids Res.*, 1989, **17**, 7273
12. Mishra, R. K., Latha, P. K. and Brahmachari, S. K., *Nucleic Acids Res.*, 1988, **16**, 4651.
13. Sambrook, J., Fritsch, E. F. and Maniatis, T., eds., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1989
14. Miller, J. H., ed, *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1972.
15. Bradford, M. M, *Anal. Biochem*, 1976, **72**, 248.
16. Singelton, C. K, *J. Biol. Chem.*, 1983, **258**, 7661.
17. Courey, A. J. and Wang, J. C., *J. Mol. Biol.*, 1988, **202**, 35.
18. Thomas, D Y., Dubuc, G. and Narang, S., *Gene*, 1982, **19**, 211.
19. Grosjean, H. and Feirs, W., *Gene*, 1982, **18**, 199.
20. Brahmachari, S K., Shouche, Y. S., Cantor, C. R. and McClelland, M., *J. Mol. Biol.*, 1987, **193**, 201.
21. Shouche, Y. S., Ramesh, N. and Brahmachari, S. K, *Nucleic Acids Res.*, 1990, **18**, 267.
22. Platt, T., *Annu. Rev Biochem.*, 1986, **55**, 339
23. Landick, R., Carey, J. and Yanofsky, C, *Proc Natl. Acad. Sci. USA*, 1987, **84**, 1507.
24. Rougive, A. E. and Lis, J. T., *Cell*, 1988, **54**, 795
25. Wright, S. and Bishop, J. M., *Proc. Natl. Acad. Sci. USA*, 1989, **86**, 505.
26. Bender, T., Thompson, C. and Kuehl, W., *Science*, 1987, **237**, 1473.
27. Piechaczyk, M, Blanchard, J. M. and Jeanteur, P., *Trends Genet.*, 1987, **3**, 47.
28. Hay, N., Skolnik-David, H and Aloni, Y., *Cell*, 1982, **29**, 183.
29. Hawley, D. K. and Roeder, R. G, *J Biol. Chem.*, 1985, **260**, 8163.
30. Muller, U. R. and Fitch, W. M., *Nature*, 1982, **298**, 582
31. Panayotatos, N. and Fontaine, A, *J Biol. Chem.*, 1987, **262**, 11364.
32. Bianchi, M. E., *EMBO J.*, 1988, **7**, 843
33. Panayotatos, N. and Wells, R. D., *Nature*, 1981, **289**, 466.
34. Pruss, G. J and Drlica, K., *Cell*, 1989, **56**, 521.
35. Liu, L. F. and Wang, J. C., *Proc. Natl. Acad Sci. USA*, 1987, **84**, 7024.
36. Boroweic, J. A, Dean, F B., Bultock, P. B. and Hurwitz, J., *Cell*, 1990, **60**, 181.

ACKNOWLEDGEMENTS. P. S S. and R. B are recipients of CSIR research fellowships. Financial support from Director, IISc, and the Department of Biotechnology towards 'Central Facility for Oligonucleotides Synthesis' is gratefully acknowledged. We thank Prof. M. R. N Murthy for helping in the development of the computer program and 'Distributive Information Centre', IISc, for providing computational facilities; Ms K. Usha and Mr P. Ramasamy for technical help and for efficient typing of the manuscript; and all the other members of S K. B.'s lab for helpful discussions

Received 31 October 1990; revised accepted 2 January 1991

RESEARCH COMMUNICATIONS

Cloning of the catalytic determinant of soluble phosphofructokinase of yeast

Z. Lobo, R. Mehta and P. K. Maitra

Tata Institute of Fundamental Research, Homi Bhabha Road, Bombay 400 005, India

The soluble phosphofructokinase of *Saccharomyces cerevisiae* is composed of two polypeptides α and β encoded by two unlinked genes *PFK1* and *PFK2* respectively. We report here cloning of the gene *PFK1* by complementation of a glucose-negative double mutant *pfk1 pfk2* using a genomic library of yeast in a multicopy vector. The anaerobic growth of the transformants on glucose is also rescued when the plasmid carries *PFK1* but not when it carries *PFK2*. The gene *PFK1* alone restores soluble-enzyme activity even if the resident *pfk2* allele is mutant. This suggests that the α subunit is the catalytic determinant of the enzyme.

GENETIC and biochemical studies on the soluble phosphofructokinase of yeast have defined two genes *PFK1* and *PFK2* involved in its synthesis. They determine, respectively, the two subunits α and β that constitute this enzyme[1-3]. Both genes have been cloned and sequenced[4,5]. Studies with a large number of mutants isolated by us have shown that mutations in *PFK1* extinguish soluble-enzyme activity without affecting aerobic growth on glucose. A second mutation in *PFK2* renders *pfk1* mutants glucose-negative[2,6,7] owing to the loss of a particulate phosphofructokinase. *pfk2* Mutants themselves retain the soluble-enzyme activity. This asymmetry in the function of the two gene products has been resolved by the observation that mutations in *PFK2* affect only the regulatory properties of the enzyme, leaving its catalytic capability intact[8]. (In the genetic nomenclature used here, *PFK1* refers to the gene for the catalytic α subunit and *PFK2* to the gene

for the regulatory β subunit, which are, respectively, 105- and 108-kDa polypeptides[5].) However, certain mutants carrying alleles of *PFK2* isolated in other laboratories gave no detectable activity *in vitro*, yet the alleles were functional *in vivo*, as shown by the profile of metabolite accumulation in intact cells[2]. This raises questions about the specific roles of these two subunits in enzyme activity. A partial answer has been obtained by studies with cloned genes[4]. elevated levels of the enzyme were seen only when both genes were present in multiple copies. Curiously, introducing the cloned *PFK1* gene alone failed to restore *in vitro* enzyme activity. We believe this is due to use of a recipient strain carrying 'atypical' *pfk2* alleles. Provision of other alleles of *pfk2* should then restitute the enzyme activity in *PFK1* transformants. We show here that a double mutant *pfk1 pfk2* can indeed be complemented for the soluble-phosphofructokinase activity by only the *PFK1* gene on a plasmid. *PFK2* clones on the other hand are without effect, although they restore growth on glucose by virtue of the particulate enzyme (Lobo and Maitra, unpublished).

Methods

Mutants in genes *PFK1* and *PFK2* isolated in this laboratory and used in this work are: *pfk1-1*, which carries a nonsense mutation[3]; and *pfk2-2* and *pfk2-4*, which carry missense mutations that affect the allosteric properties of the soluble phosphofructokinase[8]. Another mutant (gift from D. Fraenkel) is DFY70; genetic analysis indicated that it carries a mutation in the gene *PFK2* (A. Gayatri and P. K. Maitra, unpublished). The mutant allele has been designated *pfk2-70* and is referred to here as 'atypical' (see below) Genomic libraries YEp13 and YEp24 of *S. cerevisiae* in high-copy shuttle vectors were obtained through the courtesy of P. Sinha and U. Vijayraghavan. Transformation was done by the spheroplast method[9], selecting for either the ability to grow on glucose or prototrophy for the nutritional marker carried on the plasmids. Transformants were always checked for loss of markers during non-selective propagation and by retransformation. *Escherichia coli* strains MC1060 and RR1 were used for amplification of plasmid DNA. Enzyme assays were done as described[3,6] using 5 μM fructose 2,6-bisphosphate to bring out the maximum enzyme activity Cultures growing exponentially on glucose minimal medium were used for enzyme assays.

Isolation of *PFK1* and *PFK2* genes

The genes *PFK1* and *PFK2* were isolated by transforming a glucose-negative double mutant *pfk1-1 pfk2-2* to glucose positivity using, respectively, YEp13 and YEp24 genomic libraries. Either of the genes *PFK1* and *PFK2* would confer on the transformants the ability to grow on glucose by restituting, respectively, the soluble or the particulate phosphofructokinase. Purified transformant clones were tested for the soluble and the particulate enzyme. Clones carrying *PFK1* synthesized the soluble enzyme and grew in anaerobic conditions (BBL GasPak, Becton Dickinson, USA), unlike *PFK2* clones which synthesized the particulate enzyme, which is incompetent for anaerobic growth[3]. The restriction maps of the inserts are shown in Figure 1. These match the maps of *PFK1* and *PFK2* reported by Heinisch[4], but the nomenclature is reversed.

Features of complementation

The results in Table 1 show a profile of complementation of the soluble-phosphofructokinase activity using two classes of *pfk2* mutant alleles, (a) *pfk2-2* and *pfk2-4*, and (b) *pfk2-70*. As was shown earlier[8], missense mutations in *PFK2*, represented by alleles of class (a), alter only the regulatory properties of the soluble enzyme but preserve its catalytic potency. A strain of the genotype *PFK1 pfk2-70*, in contrast, gives no detectable enzyme activity in cell-free extracts (line 12), although it ferments glucose, producing fructose 1,6-bisphosphate. This has been shown to be due to the almost undetectable level of the β subunit *in vitro*[2], which in turn is due to a transcriptional defect in the mutant allele *pfk2-70*, seen in Northern blots[4]. The mutation *pfk2-70* thus approximates a null allele. Inside the cell, however, the soluble phosphofructokinase is active even in the complete absence of the β subunit as long as *PFK1* is present[10].

The double mutants *pfk1-1 pfk2-2* and *pfk1-1 pfk2-70*

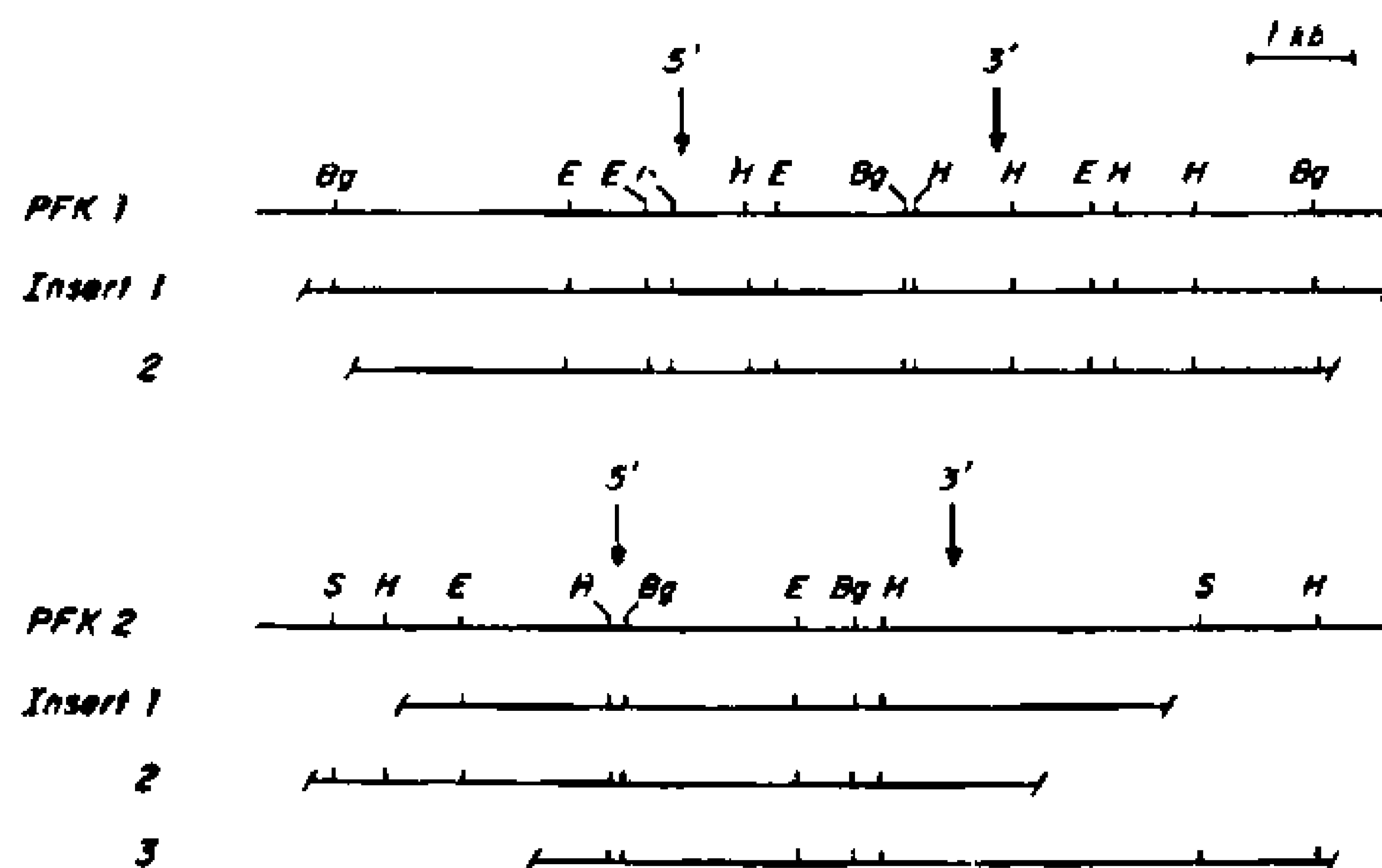


Figure 1. Restriction maps of *PFK1* and *PFK2* genes. Restriction endonucleases used were. *Bgl*II (Bg), *Eco*RI (E), *Hind*III (H) and *Sal*I (S) The arrows indicate the boundaries of the coding region, with the 5' region to the left as given in Heinisch *et al.*[5] The upper panel shows the map of two inserts carrying the *PFK1* gene and the lower panel maps of three inserts with *PFK2* Slashes denote the boundaries of the inserts

Table 1. Restoration of soluble phosphofructokinase to mutant *S. cerevisiae* by *PFK1* and *PFK2* genes in high-copy plasmids.

Line	Recipient strain genotype	Cloned gene	Growth on glucose	PFK1 activity (mU/mg protein)
1	*pfk1-1 PFK2*	—	+	0
2	*pfk1-1 pfk2-2*	—	−	0
3	*pfk1-1 pfk2-2*	*PFK1*	+	76
4	*pfk1-1 pfk2-4*	*PFK1*	+	88
5	*pfk1-1 PFK2*	*PFK1*	+	96
6	*pfk1-1 pfk2-2*	*PFK2*	+	0
7	*pfk1-1 pfk2-70*	—	−	0
8	*pfk1-1 pfk2-70*	*PFK1*	+	0
9	*pfk1-1 pfk2-70*	*PFK2*	+	0
10	*PFK1 pfk2-70*	*PFK2*	+	130
11	*pfk1-1 pfk2-2*	*PFK1 PFK2*	+	748
12	*PFK1 pfk2-70*	—	+	0

The transformants were grown under conditions maintaining the selective pressure on the particular plasmids. *PFK1* transformants from a transformation experiment using a genomic pool carried a chromosomal *leu2* marker and a plasmid-borne *LEU2*. The *PFK2* transformants, from a transformation experiment using DNA from a YEp24 library, carried *URA3* on the plasmid and *ura3* on the chromosome. The transformant carrying both *PFK1* and *PFK2* was selected by transforming a *PFK1 LEU2* transformant with a *PFK2 URA3* plasmid. PFK1 refers to the soluble phosphofructokinase.

are neither glucose-positive (growth) nor enzyme-positive (Table 1, lines 2 and 7), unlike the single mutant *pfk1-1* which is glucose-positive but lacks the soluble enzyme (line 1). The wild-type allele *PFK1* in multiple copies restores the soluble-enzyme activity of *pfk1* mutants carrying either of the mutant alleles *pfk2-2* and *pfk2-4* or the wild-type *PFK2* (lines 3–5), but not when the mutant allele *pfk2-70* is in the *pfk1* recipient strain (line 8). This is expected as the strain *PFK1 pfk2-70* itself gives no enzyme activity in cell-free extracts (line 12). The deficiency is corrected by transforming this strain with *PFK2* (line 10). Two conditions must be met for soluble-phosphofructokinase synthesis: the presence of the wild-type *PFK1* gene, together with *PFK2*, or *pfk2-2* or *pfk2-4*. The allele *pfk2-70* is clearly incompetent in this regard. The inability of Heinisch[4] to detect the soluble enzyme therefore could be due to the use of a strain carrying such an allele. The particulate phosphofructokinase being totally absent in cell-free extracts, the glucose-positive *PFK2* transformants of double mutants would appear to be enzyme-negative (lines 6 and 9) unless toluene lysates are used. The other feature of these results is the overproduction of the soluble enzyme when both *PFK1* and *PFK2* genes are present, but not when either is present singly (compare lines 3 and 6 with 11). This is expected as the soluble enzyme is an octamer of four each of the α and β subunits. These results show that the *PFK1* gene can restore the soluble enzyme in a *pfk1* mutant even if the resident *pfk2* is a missense mutant allele. The catalytic capability of soluble phosphofructokinase thus vests in the α subunit encoded by *PFK1*.

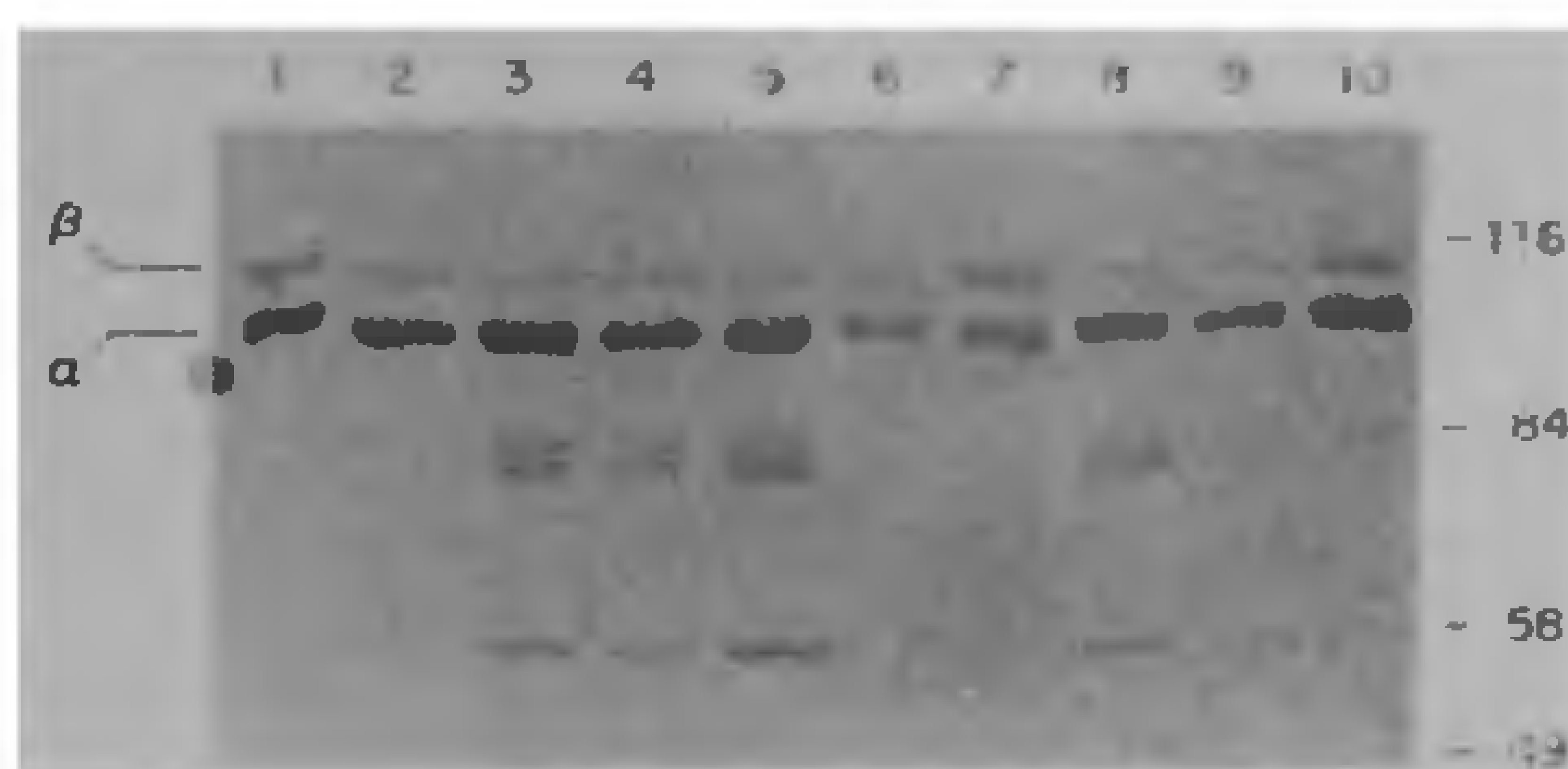


Figure 2. Immunoblot analysis of α and β subunits. Crude cell-free extracts (0.1 mg protein) from glucose-grown exponential cultures were used in all lanes except lane 1. Lane 1, 4 μg purified soluble phosphofructokinase from yeast; lane 2, wild-type strain; lane 3, *pfk1-1 pfk2-2* :: *PFK1*; lanes 4 and 5, *pfk1-1 pfk2-4* :: PFK1; lane 6, *PFK1 pfk2-2*; lane 7, *PFK1 pfk2-70* :: PFK2; lane 8, *pfk1-250 PFK2* :: PFK1; lane 9, *pfk1-1 PFK2* :: *PFK1*; lane 10, *pfk1-1 pfk2-2* :: *PFK1 PFK2*.

Analysis of subunits

To correlate the enzyme profile of the transformants with their possession or lack of the subunits of the soluble phosphofructokinase, a rabbit antibody to the purified yeast enzyme was used in immunoblotting experiments (Figure 2). Although the reaction of the antibody with the β subunit was much weaker than with the α subunit (Figure 2, lane 1), all the lanes show both bands. Cloning of the *PFK1* gene in multiple copies increases the intensity of the faster-migrating α band (lanes 3–5, 8, 9). However, this is also associated with degradation, not seen in the wild-type strain (lane 2). The degradation is less in a *PFK2*-transformant (β subunit) clone (lane 7). When both subunits are equally overexpressed (lane 10), degradation is reduced. We interpret this as indicating an inherent instability of free α subunit, i.e. when it is not associated with β. The absence of phosphofructokinase activity in cell-free extracts of the strain *PFK1 pfk2-70* is very likely due to proteolytic degradation of the catalytic α subunit in the virtual absence of any detectable β subunit[2]. Apart from imparting regulatory characteristics to the soluble enzyme, the β subunit thus provides a structural support to the catalytic subunit. A missense mutation in the *PFK2* subunit thus results in aberrant regulation of the enzyme, but preserves the scaffolding function and hence the enzyme activity *in vitro*.

1. Laurent, M., Chaffotte, A. F., Tenu, J.-P., Roucous, C. and Seydoux, F. J., *Biochem. Biophys. Res. Commun.*, 1978, **80**, 646.
2. Clifton, D. and Fraenkel, D. G., *Biochemistry*, 1982, **21**, 1935.
3. Lobo, Z. and Maitra, P. K., *J. Biol. Chem.*, 1983, **258**, 1444.
4. Heinisch, J., *Mol. Gen. Genet.*, 1986, **202**, 75.
5. Heinisch, J. *et al.*, *Gene*, 1989, **78**, 309.
6. Lobo, Z. and Maitra, P. K., *FEBS Lett.*, 1982, **137**, 279.
7. Breitenbach-Schmitt, I., Heinisch, J., Schmitt, H. D. and Zimmermann, F. K., *Mol Gen. Genet.*, 1984, **195**, 530.

8 Lobo, Z. and Maitra, P. K., *FEBS Lett.*, 1982, 139, 93.
9 Sherman, F., Fink, G. R. and Hicks, J., in *Methods in Yeast Genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1986, p. 117.
10 Heinisch, J., *Curr. Genet.*, 1986, 11, 227.

ACKNOWLEDGEMENT. The work was supported by a grant from the Department of Biotechnology, Government of India

Received 14 August 1990; revised accepted 30 October 1990

Alteration in the '−10' sequence of the A2 promoter of bacteriophage T7 reduces the rate of transcription initiation

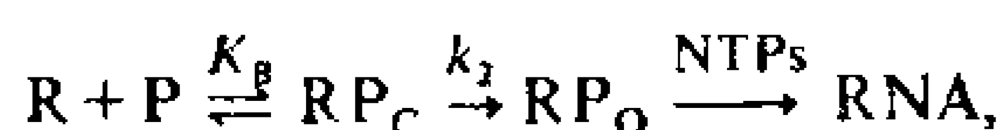
K. Prasanna Kumar, Vijaya Gopal and Dipankar Chatterji

Centre for Cellular and Molecular Biology, Hyderabad 500 007, India

The rate of transcription initiation, in the absence of other factors, is determined by intrinsic properties of the promoter. This largely depends on the DNA structure at the promoter region. The A2 promoter of bacteriophage T7 is an early promoter, specific for the RNA polymerase of the host *E. coli*. It is also one of the strongest promoters tested so far *in vitro*. We show here that a mutation in the '−10' region of T7A2 only reduces the strength of the promoter: transcription driven by the mutant promoter results in much less product. The site of initiation is the same as that in the wild-type promoter.

ANALYSIS of the various promoter sequences recognized by the major form of *E. coli* RNA polymerase, E δ^{70}, has shown that there are two highly conserved hexanucleotide sequences, TTGACA and TATAAT, centred around 35 and 10 base pairs upstream from the start point of RNA synthesis, and designated '−35' and '−10' boxes respectively[1−3]. One important feature of the promoter sequence is that it is asymmetric, and this determines the initiation of transcription in a fixed direction on the correct (template) DNA strand[4].

RNA chain-initiation frequencies in *E. coli* vary over a range of about one thousand-fold[5]. One of the important questions is how RNA polymerase recognizes the DNA sequences at the promoters around the −35 and −10 regions in a manner that results in a three-orders-of-magnitude variation in reaction rate[6]. The model proposed by Chamberlin[7] for the interaction of promoter with RNA polymerase is well accepted now, and can be depicted as follows:



$$R + P \underset{}{\overset{K_B}{\rightleftharpoons}} RP_C \overset{k_2}{\rightarrow} RP_O \xrightarrow{\text{NTPs}} RNA,$$

where R is the enzyme, P represents promoter DNA, and RP_C and RP_O designate two intermediate states, viz. the closed and open complex respectively. McClure and coworkers[6,8] correlated promoter strength with these two constants K_B and k_2, and the relationship is generally true of all the promoters studied so far.

Promoter mutation studies have indicated that the mutations affect the rate of initiation of transcription by altering either K_B or k_2. These mutations fall in the −35 or the −10 region[9,10]. These studies generally suggested that the −35 region is mainly involved in polymerase binding whereas the −10 region plays a major role in the isomerization of the closed complex to the open complex.

Bacteriophage T7 has three major early promoters A1, A2 and A3, which are recognized by the RNA polymerase of the host *E. coli*. These promoters are known to be strongest *in vitro*[6]. We have used these promoters, individually cloned in the *Bam*H1 site of pBR322 (obtained from F. W. Studier, Brookhaven National Laboratory, USA), in studies on promoter–RNA polymerase interaction[11−13]. In the presence of T7 early promoters, *E. coli* RNA polymerase transcribes almost exclusively from them, without recognizing the much weaker pBR322 promoters, even at high enzyme/DNA ratio (ref. 14, and A. Rosenberg, personal communication). When these promoters are cloned in pBR322 (Figure 1), and the plasmid digested with *Sal*1, as in the present case, the lengths of the run-off transcripts are expected to be 340 base pairs at T7A1, 310 at T7A2, and 305 at T7A3.

Of particular interest in this communication is the promoter T7A2. The sequence of the T7A2 promoter is given below:

CGAAAAACAGGTATTGACAACA
−35
TGAAGTAACATGCAGTAAGATA
−10
CAAATCGCTAGGTAACA
+1

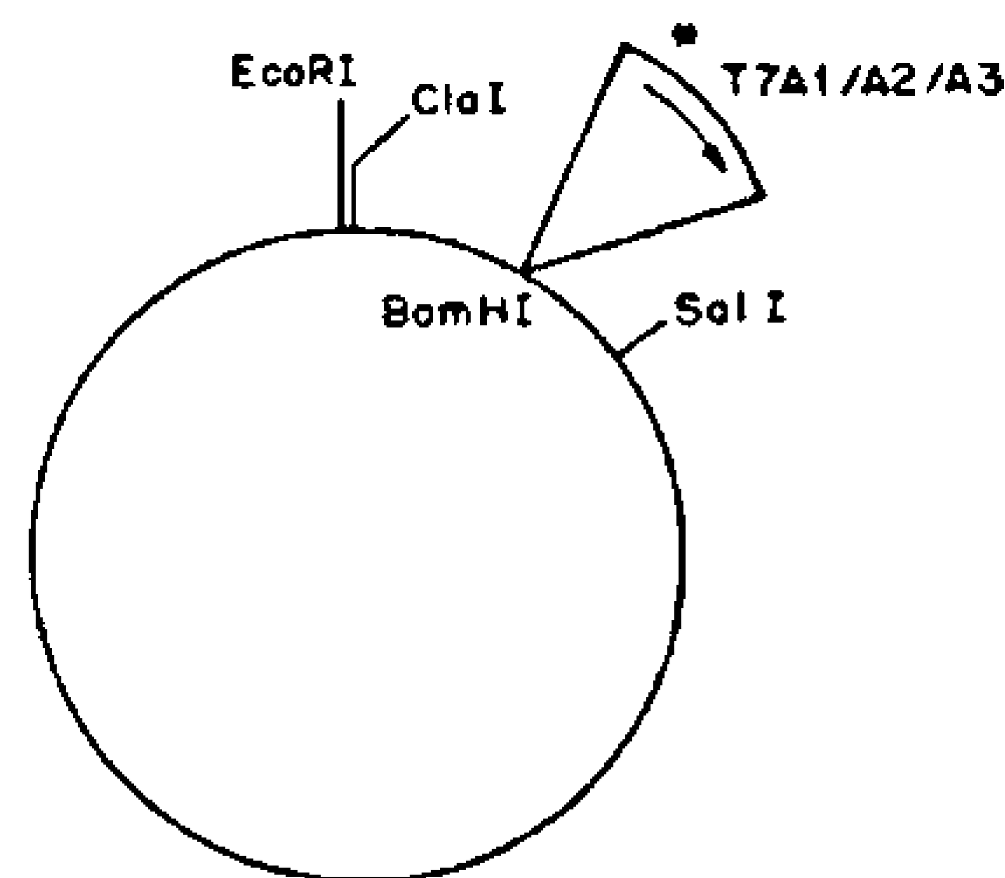


Figure 1. Diagram showing the position and orientation of the T7 promoter inserts in pBR322. The arrow indicates the direction of transcription from the promoter, which is marked by an asterisk.