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Functional complementation of yeast phosphofructokinase mutants by the non-allosteric enzyme from *Dictyostelium discoideum*

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Abstract Phosphofructokinase (PFK) from yeast has been replaced by the non-allosteric isozyme from the slime mold *Dictyostelium discoideum*. This has been achieved by overexpression of the latter in a PFK-deficient strain of *Saccharomyces cerevisiae* under the control of the *PFK2* promoter. Transformants complemented the glucose-negative growth phenotype exhibiting generation times on glucose-containing media similar to those of an untransformed strain being wild-type for yeast PFK genes. The PFK produced reacted with an antibody against *D. discoideum* PFK. It exhibited the same subunit size, quaternary structure and kinetic parameters than those of the wild-type enzyme, and was also devoid of specific regulatory properties.

Key words: Phosphofructokinase; Allosteric regulation; Glycolysis; Heterologous gene expression; *Saccharomyces cerevisiae*; *Dictyostelium discoideum*

1. Introduction

Phosphofructokinase (PFK) is a sophisticated allosteric enzyme that catalyzes the conversion of fructose-6-P into fructose-1,6-P_2 in the presence of MgATP. Its activity in most organisms is sensitive to a series of regulatory signals and is recognized as a fundamental step in the control of glycolysis [1]. Primary structures of the enzyme from various sources have been published [2–4, and references therein] and a number of amino acid residues important for catalysis and regulation were pointed out by data from 3-D structure and site-specific mutagenesis of bacterial PFKs [5–7]. Much less information has been gathered to this respect on PFK from eukaryotic cells, since it is considerably more complex in both structure and regulation and no crystal structure is available yet [8]. Nevertheless, structure–function studies based on site-directed mutagenesis [9,10] and characterization of mutations in patients with PFK deficiency [11] were lately carried out on PFK from yeast [9] and muscle [10,11].

PFK from the slime mold *Dictyostelium discoideum* (DdPFK) has been purified recently [12]. This is a non-allosteric eukaryotic PFK that, in contrast to the enzyme from other cells, shows simple kinetic properties and lacks any regulatory mechanism other than that exerted by substrate concentration [12]. DdPFK is a homotetramer of 95-kDa subunits; it has also been cloned and sequence changes with respect to regulatory isozymes have been identified at several of the putative binding sites for effectors (Estévez, A.M., Martínez-Costa, O.H., Sánchez, V. and Aragón, J.J., in prep.). PFK activity in the slime mold is scarce, ~0.5–1.2 U/g wet mass [12]. Therefore, we were interested in expressing this enzyme as a heterologous protein to obtain it in high amounts, as well as to produce mutant forms by specific manipulation of the sequence, since the peculiar properties of this PFK may be of great help in understanding the structural bases of allosteric regulation. It was of particular interest to use mutants lacking a regulated PFK for the expression of DdPFK, as this may allow us for the first time to investigate the effect of substituting a non-allosteric PFK for an allosteric one on cell physiology. Yeasts are known to be excellent host organisms for the production of foreign proteins [13–16], including genes from *D. discoideum* [17–19]. More specifically, PFK isozymes from *Escherichia coli* and muscle have been successfully expressed in *Saccharomyces cerevisiae* [20]. This yeast owns PFK activity controlled by various allosteric effectors [21]. The enzyme is an octamer composed of two types of subunits, α and β [22], which are encoded by *PFK1* and *PFK2*, respectively [4]. Simultaneous inactivation of both genes leads to a lack of the ability to grow on glucose as a sole carbon source [23]. In this work, we report the overexpression of DdPFK in a double *S. cerevisiae* mutant carrying deletions in both PFK genes. The recombinant enzyme is active in vivo and in vitro and has been purified and characterized. The effect of replacement of the highly regulated PFK from yeast by the non-allosteric *D. discoideum* enzyme on cell growth is also reported.

2. Materials and methods

2.1. Materials

Restriction enzymes, T4 DNA ligase and deoxynucleotides were obtained from Boehringer Mannheim. *Taq* DNA polymerase was from Beckman Instruments. RNase, auxiliary enzymes used in the assay for PFK, phosphoric esters, ATP and NADH were purchased from Sigma. The Geneclean kit was obtained from Bio 101 and the Sequenase 2.0 sequencing kit from United States Biochemicals. Oligonucleotides were purchased from MedProbe. Other reagents were obtained from commercial sources and were of the best grade available.

2.2. Strains and media

E. coli strain DH5α [24] was used for plasmid propagation and isolation. *S. cerevisiae* strain HD114–8D (*MATα pfk1::HIS3 pfk2::HIS3 ura3–52 leu2–3 his3–11,15*) [11], carrying deletions in both yeast PFK genes *pfk1* and *pfk2*, was used as a recipient strain for expression of DdPFK. It was grown in either rich media (1% yeast extract, 1% bacto peptone, 2% glucose; YEPD) or minimal media (YNB) containing 0.67% yeast nitrogen base supplemented with amino acids as described [25]. As carbon sources, 2% glycerol plus 2% ethanol (YNBGE) or 2% glucose (YNBD) were added. Generation times were measured by following the OD_{600}.

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2.3. Genetic manipulations, plasmids and sequencing

Analysis, construction and purification of DNA followed standard techniques [26]. Yeast cells were transformed by the procedure described by Klebe et al. [27] as modified by Dohmen et al. [28]. The custom-made oligonucletide 5′-AGATCTGGATCCATGACAAC-AACGTAAAAATCATT-3′ (primer A), bearing a *Bam*HI site, was used for construction of a fusion directed at the ATG initiation codon in conjunction with the reverse primer for pBluescript to amplify the DdPFK cDNA (a 2.7-kb *Bam*HI/*Acc*I fragment from pE2; see below) by PCR. The amplification reaction was performed by mixing 50 ng of template DNA, 0.2 mM each deoxyribonucleside triphosphate, 500 ng of sense and antisense primers, 2.5 U of *Taq* DNA polymerase and the buffer provided by the manufacturer in a total volume of 100 μl. The PCR was carried out for 25 cycles (94°C, 1 min; 45°C, 2 min; 72°C, 5 min). The resulting fragment was isolated from an agarose gel using the Geneclean kit, digested with *Bam*HI and cloned into pUC19 linearized with *Bam*HI to give pE3 (Fig. 1). For expression in yeast, the *Bam*HI fragment from pE3 was cloned into the plasmids pJJH70 and pJJH71 digested with the same restriction enzyme to yield pEHA3 and pEHA1, respectively. The plasmids pJJH70 and pJJH71 contain the *PFK2* promoter on an *Eco*RI/*Bam*HI fragment [11]. pJJH70 is based on the yeast/*E. coli* shuttle vector pBM272 with a CEN/ARS element ensuring replication and propagation in yeast [20]. The *Eco*RI/*Bam*HI fragment of the latter, originally carrying the *GAL1–10* promoter, was substituted by the *PFK2* promoter fragment. For construction of pJJH71, the *PFK2* promoter fragment was cloned into the multiple cloning site of the multicopy vector YEp352 [29], digested with *Eco*RI/*Bam*HI. Sequence determination, to ensure that no errors were introduced by PCR, was based on the dideoxynucleotide chain-termination method [30] using the Sequenase 2.0 sequencing kit as outlined in the manufacturer's manual.

2.4. Enzymatic and immunological analysis

PFK activity was measured by using a spectrophotometric coupled assay for the formation of fructose-1,6-P_2 as described previously [12]. When PFK activity was measured during yeast growth, samples of cell culture were washed with buffer A (50 mM Hepes, 100 mM KCl, 2 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 2 mM NaF, 2.5 μg leupeptin, pH 6.8). The cell pellet was resuspended in 0.75 ml of the same buffer, mixed with 0.5 ml glass beads (0.5-mm diameter) and vigorously shaken by vortexing for four 1-min periods at 1-min intervals. During the intervals, the tubes were kept on ice. The extracts were then centrifuged at 13,000 × *g* for 10 min at 4°C and PFK activity was measured in the supernatant. One unit of activity is defined as the amount that catalyzes the conversion of 1 μmol of substrate at 25°C.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% acrylamide gels, Western blot analysis and immunostaining were performed as described previously [12]. Protein was determined by the method of Bradford [31] with bovine γ-globulin as standard.

2.5. Purification of recombinant DdPFK

Yeast transformants expressing DdPFK were grown in 1 liter of YEPD to early stationary phase, harvested by sedimentation and washed in buffer A. ~10 g wet cells were resuspended in 2 vol. buffer A, mixed with 5 vol. glass beads and shaken in a refrigerated mill for 5 min. After filtration, the beads were washed with 1 vol. buffer A. The filtrate was centrifuged at 31,000 × *g* for 30 min. Protamine sulfate dissolved in buffer A was added to the supernatant to a final concentration of 0.2% (w/v). After 30 min stirring the mixture was centrifuged as before. The supernatant fluid was collected and designated as extract. PFK activity was purified from the extract by fractionation between 5 and 10% (w/v) polyethylene glycol and chromatography on DE52 and blue-Sepharose CL-6B as described previously [12] for the purification of the enzyme from *D. discoideum* except that the elution buffer of the blue-Sepharose column contained 1.5 mM MgATP and 3 mM fructose-6-P. Fractions having the highest activity were combined, dialyzed against the column equilibration buffer and employed in subsequent studies. The final purified fraction had a specific activity of 61.6 U/mg protein measured with 1 mM fructose-6-P and 1 mM MgATP, pH 7.2.

2.6. Non-denaturing agarose gel electrophoresis of PFK

Horizontal slab electrophoresis on 0.7% agarose gels was performed as described by Li et al. [10] except that the buffer used for gel preparation and electrophoresis development was 30 mM Tris-phosphate, pH 8.0. PFK samples were dialyzed against 30 mM Tris-phosphate, pH 8.0 and mixed with 0.25 vol. of the same buffer containing 25% (v/v) glycerol and 0.25% Bromophenol blue before loading. After electrophoresis, the proteins were blotted onto nitrocellulose paper by capillary transfer and PFK was detected by immunostaining as above.

3. Results and discussion

3.1. Construction of expression plasmids

Expression of DdPFK was first attempted in *E coli* cells without success. Thus, cloning of the DdPFK cDNA as a 2.7-kb fragment into the plasmids pUC18 or pMAL (New England Biolabs), followed by transformation of the PFK-deficient *E. coli* strain DF1020 and expression under the control of the *lac* promoter did not lead to an active PFK, since the protein was highly degraded as detected by Western blotting (data not shown). Cloning of the cDNA into the plasmid pT7–7 [32] under the T7 RNA polymerase promoter and transformation of *E. coli* strain BL21(DE3) gave similar results. Therefore, we resorted to the yeast *S. cerevisiae* which has been reported to be a good alternative for heterologous expression of PFK genes (see section 1).

Two expression plasmids, pEHA3 and pEHA1, were constructed that contained the *Bam*HI DdPFK cDNA fragment inserted downstream of the *PFK2* promoter of *S. cerevisiae* in plasmids pJJH70 and pJJH71, respectively (Fig. 1). The initial DdPFK cDNA insert (from plasmid pE2; Estévez, A.M., Martínez-Costa, O.H., Sánchez, V. and Aragón, J.J., in prep.) contained the entire coding sequence of DdPFK (accession number X89039) and 260 bp of the 3′-non-coding region of the originally isolated cDNA, but lacked the ATG initiation codon. Therefore, a new *Bam*HI site and an ATG codon were fused at the 5′-end by PCR (see Section 2). The DdPFK cDNA was then cloned as a *Bam*HI fragment into the yeast vectors pJJH70 (pEHA3) and pJJH71 (pEHA1), as shown in Fig. 1.

3.2. Expression in a yeast pfk1 pfk2 double mutant

Yeast strains lacking their endogenous *PFK* genes do not grow on glucose-containing media. To test if this phenotype can be complemented by our constructs, plasmids pEHA3 and pEHA1, as well pJJH70 and pJJH71 which do not carry the DdPFK cDNA, were used to transform the yeast strain

Table 1
Purification of *D. discoideum* phosphofructokinase expressed in yeast

Step	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg of protein)	Purification (fold)	Yield (%)
Extract	25	282.3	322.5	1.1	1	100
Polyethylene glycol 5–10%	5.5	84.5	108.9	1.3	1	34
DE52 chromatography	14	4.3	57.2	13.3	12	18
Blue-sepharose chromatography	6	1.1	67.8	61.6	56	21

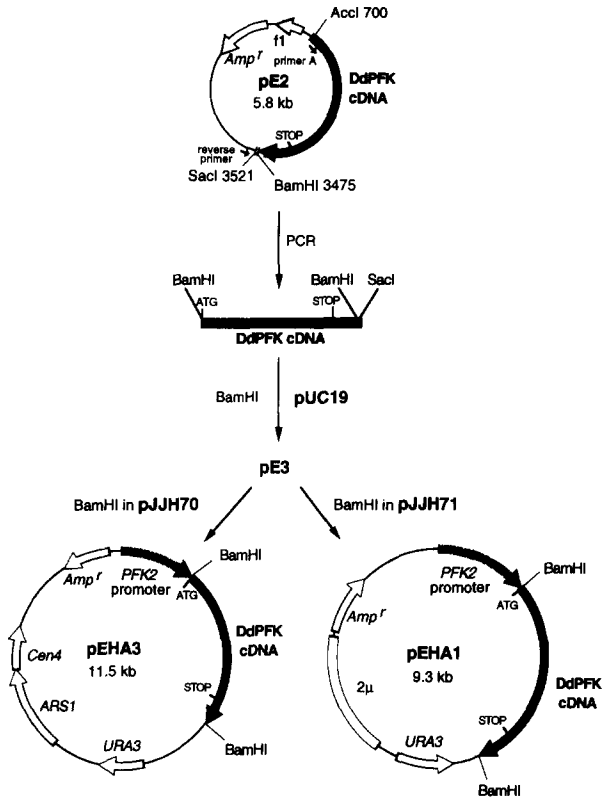

Fig. 1. Construction of plasmids for the expression of *D. discoideum* phosphofructokinase cDNA in yeast. Relevant genes are indicated by broad arrows. Arrow heads point in the direction of transcription.

HD114–8D (*pfk1::HIS3 pfk2::HIS3 ura3–52*). Transformants were first selected on YNBGE without uracil. Three clones obtained with each plasmid were tested for their ability to grow on glucose. As shown in Fig. 2, only transformants with plasmids bearing DdPFK cDNA, pEHA3 and pEHA1, complemented for growth. We take that as strong evidence that DdPFK is functionally expressed in yeast.

Growth of transformants with pEHA1 in liquid YEPD medium led to a steady increase of ~2-fold in PFK activity (Fig. 3A) that was practically paralleled by a similar increment in the amount of recombinant protein, as detected by Western blot (Fig. 3B), during the early growth phase. Both reached a maximum after ~24 h. The PFK produced exhibited an electrophoretic mobility identical with that of the immunoreactive band corresponding to the wild-type *D. discoideum* enzyme. No immunological reactivity was observed in extracts from transformants with the plasmid pJJH71, showing further that the expression was specifically related to the presence of DdPFK. PFK activity in transformants with pEHA1 was 6× higher than with pEHA3. Although 2-μm vectors are expected to be present at 20–30 copies/cell, this result coincides with overexpression of the homologous yeast PFK genes [23]. There, too, only a 3–5× increase in specific activities was observed upon expression from such vectors. Therefore, we chose the multicopy plasmid for subsequent studies. Transformants with pEHA1 grew on YEPD with generation times of 116 min, which was similar to that exhibited by an isogenic, untransformed strain being wild-type for the yeast PFK genes. Therefore, replacement of the highly regulated PFK from yeast [21] by a non-allosteric one, such as that from *D. discoideum* [12], apparently does not affect the ability of yeast to grow on glucose.

Table 2
Steady-state kinetic parameters of both wild-type (W) and recombinant (R) *D. discoideum* phosphofructokinase

Substrate	$S_{0.5}$ (mM)		n_H		V_{max} (U/mg of protein)	
	W	R	W	R	W	R
Fructose-6-P	22	24	1.05	1.08	89	76
MgATP	16	19	1.1	1.0	89	76

Reaction was started by addition of fructose-6-P. With fructose-6-P as substrate MgATP concentration was 2.5 mM; with MgATP as substrate, fructose-6-P concentration was 1 mM. Values corresponding to wild-type PFK were taken from Martínez-Costa et al. [12].

3.3. Purification and properties of the recombinant enzyme

PFK activity was over 200× higher in a cell extract from the transformed strain (1.1 U/mg protein) than in that from *D. discoideum* amoebae (0.005 U/mg protein [12]), being therefore amenable to a relatively simple purification. Recombinant DdPFK was then purified from yeast transformants in three steps by the procedure summarized in Table 1 with a final yield of 21%. The final preparation had a specific activity of 61.6 U/mg, which is close to that of the enzyme from the slime mold (83.5 U/mg [12]) and was judged to be homogeneous by the criteria of SDS-PAGE (Fig. 4A). Its subunit size appeared identical with that of the wild-type enzyme, 95 kDa [12]. Both types of enzyme behaved also similarly on non-denaturing agarose gel electrophoresis (Fig. 4B), thus, suggesting that the recombinant enzyme has the same quaternary structure, i.e. a tetramer of identical subunits [12]. From 1 liter of culture, 1.1 mg of pure DdPFK was obtained. The enzyme can be stored at −20°C in the presence of 3 mM fructose-6-P and 1.5 mM MgATP for at least 3 months without apparent loss of activity. The amount of recombinant enzyme in yeast transformants was calculated to represent 1.9% of total extract protein. This value is nearly 300× higher than in *D. discoideum* cells (6.5×10^{-3}% [12]). The enzyme was also overexpressed with respect to native yeast PFK, since the later is ~0.5% of extract protein [33,34]. After completion of this work, we observed that the recombi-

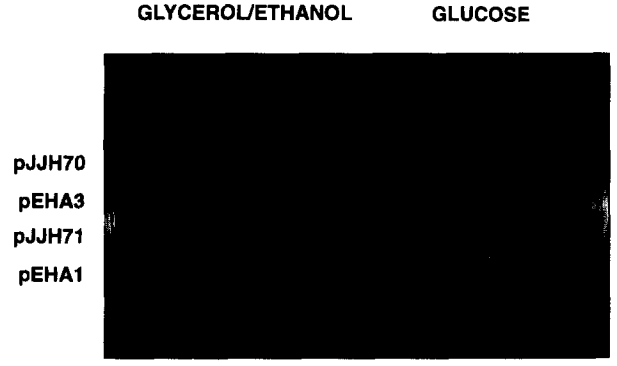

Fig. 2. Growth of transformants on different carbon sources. Three independent transformants of strain HD114–8D (*pfk1::HIS3 pfk2::HIS3 ura3–52*) with the plasmids indicated (also compare Fig. 1) were streaked onto YNBGE, left to grow and replica-plated onto YNBD lacking uracil. They were incubated for 3 days at 30°C.

nant enzyme can also be obtained pure by omitting the polyethylene glycol fractionation step (Table 1), although with a yield of only 7%.

As shown in Table 2, DdPFK expressed in yeast exhibits virtually the same kinetic properties as those of the wild-type enzyme, i.e. a high affinity for both fructose-6-P ($S_{0.5}$ = 24 μM) and MgATP ($S_{0.5}$ = 19 μM) and a hyperbolic saturation curve (n_H = 1.08) with respect to fructose-6-P. This enzyme was also devoid of regulatory characteristics, such as ATP inhibition and fructose-2,6-P_2 activation or sensitivity to other typical allosteric effectors of PFK from other cells (P_i, AMP, NH_4^+, citrate, fructose-1,6-P_2, P-enolpyruvate; data not shown).

3.4. Conclusions

The results presented here show that PFK from *D. discoideum* has been functionally overexpressed in a PFK-deficient strain of *S. cerevisiae* under the control of the *PFK2* promoter. The recombinant enzyme was purified to homogeneity; it exhibits the same structural, immunological and kinetic properties than the wild-type enzyme and has been produced in sufficient amounts for studies of both wild-type and mutant DdPFK. Additionally, these results represent the first report of substituting a non-regulated PFK for an allosteric one. Yeast PFK is an octamer composed of two different subunits [22] endowed with sophisticated regulatory properties [21]. However, expression of the heterologous DdPFK, a homotetrameric, non-allosteric isozyme [12], in a yeast PFK double mutant elicited an apparently normal growth of transformants on glucose. Therefore, our work raises questions on the contribution of the regulatory behavior of yeast PFK observed in vitro to the control of the flux from fructose-6-P to fructose-1,6-P_2 in vivo in glucose-consuming yeast. Further studies to clarify this important point are in progress.

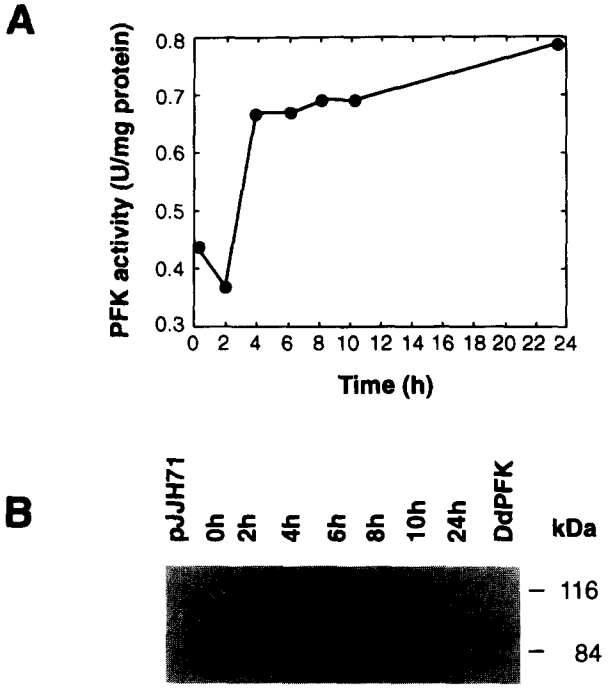

Fig. 3. Phosphofructokinase expression during yeast growth. (A) Strain HD114-8D (*pfk1::HIS3 pfk2::HIS3 ura3-52*) transformed with pEHA1 (Fig. 1) was grown in 50 ml of YEPD to early stationary phase, washed with sterile water and inoculated in fresh medium at about half the original cell density at time 0. At the time points indicated, samples of 8 ml were taken from the growth medium and cell extracts were prepared for determination of PFK activity. (B) Western blot with 50 μg of protein of the cell extracts prepared in (A) loaded in each lane. The recipient strain transformed with pJJH71 was used as a negative control (pJJH71). Wild-type DdPFK, partially purified up to the step of blue-Sepharose chromatography as described by Martínez-Costa et al. [12], was used as a control (DdPFK). Positions of relevant molecular mass markers are shown on the right.

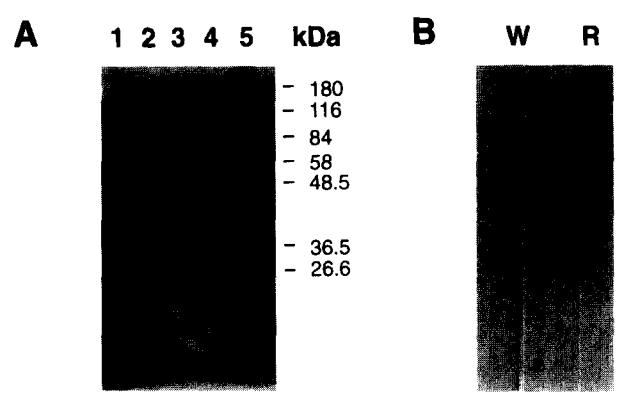

Fig. 4. Electrophoretic analysis of recombinant PFK. (A) SDS-PAGE at various stages of purification of recombinant phosphofructokinase. Samples obtained during purification were analyzed by SDS-PAGE and silver-stained. Lanes: 1, extract (50 μg protein); 2, polyethylene glycol, 5–10% (30 μg protein); DE52 chromatography (20 μg protein); 4, blue-Sepharose chromatography (10 μg protein). Positions of molecular markers (in kilodaltons) are shown on the right. (B) Non-denaturing agarose gel electrophoresis of both wild-type (W) and recombinant (R) *D. discoideum* phosphofructokinase. After electrophoresis, PFK was detected by Western blotting.

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References

[1] Sols, A. (1981) Curr. Top. Cell. Regul. 19, 77–101.
[2] Lee, C.-P., Kao, M.-C., French, B.A., Putney, S.D. and Chang, S.H. (1987) J. Biol. Chem. 262, 4195–4199.
[3] Rongnoparut, P., Verdon, C.P., Gehnrich, S.C. and Sul, H.S. (1991) J. Biol. Chem. 266, 8086–8091.
[4] Heinisch, J., Ritzel, R.G., Von Borstel, R.C., Aguilera, A., Rodicio, R. and Zimmermann, F.K. (1989) Gene 78, 309–321.
[5] Schirmer, T. and Evans, P.R. (1990) Nature (London) 343, 140–145.
[6] Auzat, J., Le Bras, G. and Garel, J.-R. (1994) Proc. Natl. Acad. USA 91, 5242–5246.
[7] Byrnes, W.M., Hu, W., Younathan, E.S. and Chang, S.H. (1995) J. Biol. Chem. 270, 3828–3835.
[8] Poorman, R.A., Randolph, A., Kemp, R.G. and Heinrikson, R.L. (1984) Nature (London) 309, 467–469.
[9] Arvanitidis, A. and Heinisch, J.J. (1994) J. Biol. Chem. 269, 8911–8918.
[10] Li, J., Zhu, X., Byrnes, M., Nelson, J.W. and Chang S.H. (1993) J. Biol. Chem. 268, 24599–24606.
[11] Raben, N, Exelbert, R., Spiegel, R., Sherman, J.B., Nakajima, H., Plotz, P. and Heinisch, J. (1995) Am. J. Hum. Genet. 56, 131–141.
[12] Martínez-Costa, O.H., Estévez, A.M., Sánchez,V. and Aragón, J.J. (1994) Eur. J. Biochem. 226, 1007–1017.
[13] Buckholz, R.G. and Gleeson, M.A.G. (1991) Biotechnology 9, 1067–1072.

[14] Heinisch, J.J. and Hollenberg, C.P. (1993) in: Biotechnology, Vol. 1 (Rehm, H.-J. and Reed, G., Eds.), pp. 469–514, VHC Verlagsgesellschaft, Weinheim, Germany.
[15] Centeno, F., Deschamps, S., Lompré, A.-M., Anger, M., Mountin, M.-J., Dupont, Y., Palmgren, M.G., Villalba, J.M., Møller, J.V., Falson, P. and Le Maire, M. (1994) FEBS Lett. 354, 117–122.
[16] Proudfoot, A.E.I., Turcatti, G., Wells, T.N.C., Payton, M.A. and Smith, D.J. (1994) Eur. J. Biochem. 219, 415–423.
[17] Shi, N.Q. and Thornburg, R. (1993) Gene 127, 199–202.
[18] Schricker, R., Magdolen, V., Kaniak, A., Wolf, K. and Bandlow, W. (1992) Gene 122, 111–118.
[19] Marschalek, R., Kalpaxis, D. and Dingermann, T. (1990) EMBO J. 9, 1253–1258.
[20] Heinisch, J.J. (1993) FEBS Lett. 328, 35–40.
[21] Gancedo, C. and Serrano, R. (1989) in: The Yeasts, Vol. 3 (Rose, A.H. and Harrison, J.S., Eds.), pp. 205–259, Academic Press, New York, NY.
[22] Kopperschläger, G., Bär, J., Nissler, K. and Hofmann, E. (1977) Eur. J. Biochem. 81, 317–325.
[23] Heinisch, J.J. (1986) Curr. Genet. 11, 227–234.
[24] Hanahan, D. (1983) J. Mol. Biol. 166, 557–580.
[25] Zimmermann, F.K. and Scheel, I. (1977) Mol. Gen. Genet. 154, 75–82.
[26] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[27] Klebe, R.J., Harris, J.V., Sharp, Z.D. and Douglas, M.G. (1983) Gene 25, 333–341.
[28] Dohmen, R.J., Strasser, A.W.M., Höner, C.B. and Hollenberg, C.P. (1991) Yeast 7, 691–692.
[29] Hill, J.H., Myers, A.M., Koerner, T.J. and Tzagoloff, A. (1986) Yeast 2, 163–167.
[30] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. USA 74, 5463–5467.
[31] Bradford, M. M. (1976) Anal. Biochem. 72, 248–254.
[32] Tabor, S. (1994) in: Current Protocols In Molecular Biology. (Ausubel, F.M. et al., Eds.) pp. 16.2.1–16.2.11, Wiley Interscience, New York, NY.
[33] Hofmann, E. and Kopperschläger, G. (1982) Methods Enzymol. 90, 49–60.
[34] Chaffotte, A.F., Laurent, M., Tijane, M., Tardieu, A., Roucos, C., Seydoux, F. and Yon, J.M. (1984) Biochemie 66, 49–58.