Vol. 176, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 15, 1991 Pages 137-144

EXPRESSION OF RAT LIVER FRUCTOSE-1,6-BISPHOSPHATASE IN *ESCHERICHIA COLI*

M. Raafat El-Maghrabi and Simon J. Pilkis

Department of Physiology and Biophysics
State University of New York at Stony Brook, NY 11794

Received January 31, 1991

SUMMARY: Rat liver fructose-1,6-bisphosphatase was expressed in *Escherichia coli* using a T7 RNA polymerase-transcribed expression system. Maximum yields of soluble active enzyme were obtained when the bacterial host cell, BL21(DE3), carrying the expression plasmid was grown and transcription induced in LB medium at 37°C. Approximately 20mg of fructose-1,6-bisphosphatase are synthesized per liter of culture after 4hr, of which about 10mg are soluble and enzymatically active. Expressed fructose-1,6-bisphosphatase, purified to homogeneity by substrate elution from a carboxymethyl Sephadex column, was indistinguishable from that purified from rat liver in terms of subunit size and kinetic properties. The *in vitro* expression of fructose-1,6-bisphosphatase in an heterologous system is a necessary preliminary step for future studies on site-directed mutant enzyme forms.

The regulation of fructose-1,6-bisphosphatase (EC 3.1.3.11, Fru-1,6-P_2ase) activity is essential in tissues such as liver and kidney that are capable of switching from net glucose consumption, or glycolysis, to net glucose synthesis, or gluconeogenesis. Inhibition of this key gluconeogenic enzyme during periods of enhanced glycolysis is primarily due to the presence of Fru 2,6-P_2 (1,2). Micromolar concentrations of Fru 2,6-P_2 inhibit Fru-1,6-P_2ase activity, and also potentiate the allosteric inhibition of the enzyme by AMP. The mechanism of inhibition of Fru-1,6-P_2ase by Fru 2,6-P_2 has been extensively studied (3-5) but remains controversial. Some of the amino acid residues responsible for substrate and effector binding have been tentatively identified either by chemical modification studies (reviewed in 6) or by x-ray crystallographic analyses of the pig kidney enzyme (7). Definitive identification of

Abbreviations: Fru-1,6-P_2ase, fructose-1,6-bisphosphatase; IPTG, isopropyl-β-D-thio-galactopyranoside; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; and Fru-1,6-P_2, fructose-1,6-bisphosphate; SDS-PAGE, sodium dodecyl polyacrylamide gel electrophoresis.

0006-291X/91 $1.50

these residues can only be accomplished by the expression of sufficient amounts of native and mutant enzyme forms in a heterologous expression system. We have recently reported the sequence of a coding-length cDNA to rat liver Fru-1,6-P_2ase as well as the complete amino acid sequence of the enzyme (8). This report describes the construction of a plasmid expression vector using the the rat liver Fru-1,6-P_2ase cDNA, the expression in *E. coli* of significant levels of soluble, enzymatically active Fru-1,6-P_2ase using this expression plasmid, and a rapid purification scheme that yields homogeneous enzyme.

MATERIALS AND METHODS

Materials - The pET3a expression vector containing the ϕ10 promoter for T7 RNA polymerase and the bacterial host cell strains BL21(DE3) ± pLysS were kind gifts of Dr. William Studier (Brookhaven National Labs). Restriction enzymes were from New England Biolabs, and T4 DNA ligase and polynucleotide kinase were from Boehringer as were yeast glucose-6-phosphate-dehydrogenase and phosphoglucose-isomerase. Isopropyl-β-D-thio-galactopyranoside was from Sigma and L-[^{35}S]methionine (1061 Ci/mmol) was from Du Pont. Oligodeoxynucleotides were synthesized on an Applied Biosystems Model 380A synthesizer and purified on OPC™ cartridges from the same company.

Construction of the Fru-1,6-P_2ase Expression Plasmid - The expression of Fru-1,6-P_2ase was accomplished using the T7 RNA polymerase-based system of Studier and Moffat (9). The expression plasmid (pFBPET) was constructed from an AsuII/XbaI fragment of the rat liver Fru-1,6-P_2ase cDNA (encoding amino acids 7 to 360, 8) and two pairs of complementary oligonucleotide linkers (see Fig. 1). The 5' linkers (NdeI/AsuII), encoding the N-terminal 6 amino acids and the translation initiation site were phosphorylated, annealed and ligated to the NdeI site of the pET3a vector (10) and the AsuII site of the cDNA. The 3' linkers (XbaI/EcoRI), encoding the C-terminal phenylalanine and the translation termination site, were similarly treated and ligated to the XbaI site of the cDNA and to the EcoRI site of pET3a. The authenticity of all plasmid constructs and the fidelity of the entire coding sequence of the Fru-1,6-P_2ase gene were determined by dideoxy chain termination sequencing with modified T7 DNA polymerase (US Biochemicals). The bacterial host cell strains, *Escherichia coli* BL21(DE3) ± pLysS each contain a single integrated copy of the T7 RNA polymerase gene controlled by the IPTG-inducible lac UV5 promoter (9,10). The cells were transformed with pFBPET and the transformed cells were selected on LB plates containing ampicillin (100μg/ml) ± chloramphenicol (30μg/ml), respectively.

Growth and induction - Bacterial strains carrying the expression plasmid were grown at 37°C in M9 medium containing ampicillin (100μg/ml) and, in the case of the pLysS-containing strain, chloramphenicol (30μg/ml). When the A_{600nm} reached ~1, two aliquots of the cells (100μl and 1ml) were saved for "zero-time" samples, IPTG was then added to the remainder of the culture to a final concentration of 1mM and the incubation continued. Two aliquots were removed after 30min, 1, 2, 4, 8 and 16hr, and the cells of all samples were harvested by centrifugation.

Estimation of Induced Fru-1,6-P_2ase - Cells from the 100μl samples were suspended in 25μl of SDS-PAGE loading buffer denatured at 90°C for 5min and electrophoresed on 10% polyacrylamide gels as described by Laemmli (11). Total Fru-1,6-P_2ase protein was quantitated by comparing the Coomassie Blue-stained bands on the gel with purified rat liver Fru-1,6-P_2ase as standard.

Rate of [^{35}S]Methionine Incorporation - Rates of protein synthesis were determined by preincubating 100μl of the cells for 3min at 37°C with 1.5μCi of [^{35}S]methionine before harvesting and electrophoresis. The stained electrophoretograms were exposed to X-ray film for 5hr and the autoradiograms quantitated by densitometry scanning.

Assay of Fru-1,6-P_2ase - To assay expressed Fru-1,6-P_2ase, cells from the 1ml aliquots were suspended in 0.1ml Fru-1,6-P_2ase extraction buffer (20mM KP_i, pH 7.5, 1mM EDTA, 1mM EGTA, 1mM DTT, 0.5mM PMSF, 2.5µg/ml leupeptin and 1mg/ml lysozyme), and subjected to 3 cycles of freezing (dry ice/ethanol bath) and thawing. The extract was made 10mM in $MgSO_4$, 5µg of DNase I was added and the extract placed on ice for 1h and then centrifuged at 10,000 x g for 10min. The supernates were assayed for Fru-1,6-P_2ase, spectrophotometrically, by coupling the production of fructose-6-phosphate to the reduction of $NADP^+$ using phosphoglucose-isomerase and glucose-6-phosphate-dehydrogenase as previously described (12).

Other methods - Protein concentration was determined according to Lowry et al (13) and protein sequencing was performed as previously described (14).

RESULTS

Construction of the Fructose-1,6-bisphosphatase/pET expression vector. Figure 1 is a schematic representation of the Fru-1,6-P_2ase expression plasmid construct, pFBPET. The 5'-pair of oligonucleotide linkers provide an NdeI restriction site and also restore the coding sequence for the N-terminal 6 amino acids of the Fru-1,6-P_2ase cDNA (8). Similarly, the 3'-pair of linkers encode the C-terminal phenylalanine, the translation termination codon and an EcoRI site. The NdeI/EcoRI cDNA fragment (1089 bp) was then ligated to the NdeI, EcoRI-digested pET3a vector, immediately following the ϕ10 promoter of T7 RNA polymerase (10) (Fig.1).

Induction of Enzymatically Active Fructose-1,6-bisphosphatase. The expression plasmid was tested for its ability to produce Fru-1,6-P_2ase after induction of the T7 RNA polymerase gene. A time course of IPTG induction of Fru-1,6-P_2ase in BL21(DE3) cells as monitored by SDS-PAGE is shown in Fig. 2. As early as 30min after IPTG addition there

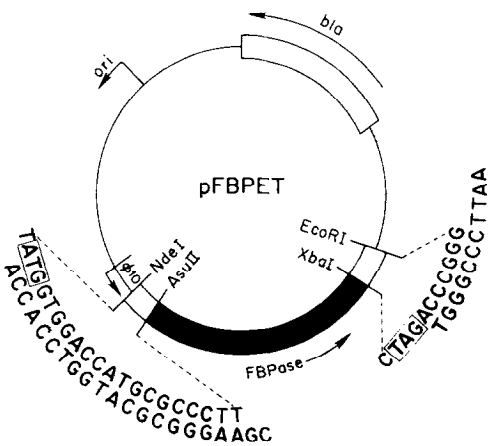


Figure 1. Construction of the Fru-1,6-P_2ase Expression Plasmid. The Fru-1,6-P_2ase expression plasmid, pFBPET, was constructed from the cDNA for the enzyme, a pET3a vector and two pairs of oligonucleotide linkers as described under "Materials and Methods". The sequences of the oligonucleotide linkers are shown. *Ori* and *bla* are the locations of the origin of replication and the β-lactamase gene, respectively, and ϕ10 is the T7 promoter region.

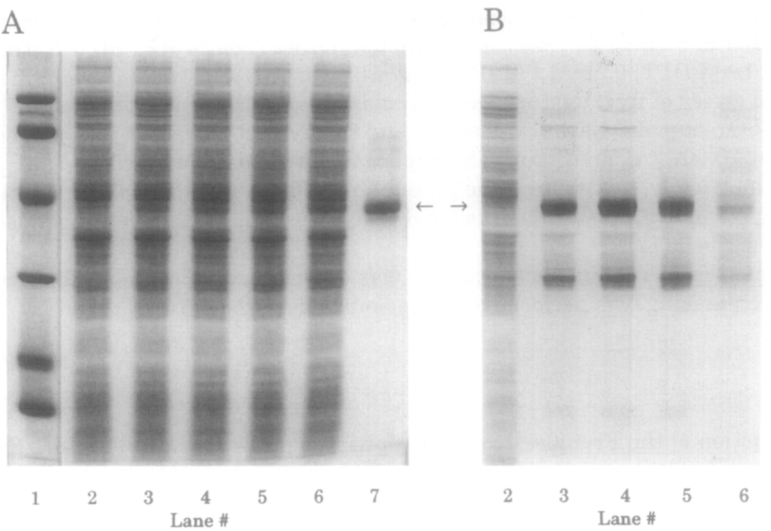


Figure 2. Time course of IPTG Induction of pFBPET. Samples from a time course of IPTG induction of pFBPET in *E.coli* BL21(DE3), grown in M9 medium, were pulse-labelled with [^{35}S]Met and subjected to SDS-PAGE as described under "Materials and Methods". A. is the Coomassie Blue stained gel. B. is an autoradiogram of A. Lane 1, marker standards, in decreasing order 94kDa, 66kDa, 45kDa, 31kDa, 21kDa and 14kDa. Lanes 2 to 6 are zero h, 0.5h, 1h, 2h and 4h samples after IPTG induction and lane 7 is Fru-1,6-P2ase purified from rat liver.

was an accumulation of a 40kDa peptide that migrated with the same mobility as purified rat liver Fru-1,6-P_2ase (Fig. 2A, lane 7). The amount of expressed protein reached a maximal level by 4h (Fig. 2A) and was reduced thereafter (data not shown). Pulse-chase labelling of induced protein with ^{35}S-methionine shows that the rate of Fru-1,6-P_2ase synthesis peaked at about 1hr, at which time the enzyme was the major synthesized protein, representing about 70% of all newly synthesized protein (Fig. 2B). Of the latter, β-lactamase (Fig. 2B, 30kDa band), the expression of which is also induced by IPTG (9,10), constituted the remaining 30%.

In order to determine how much of the expressed enzyme was soluble, the cells were first lysed and the lysate separated into soluble and insoluble fractions before electrophoresis. Approximately 50% of the total amount of expressed enzyme was present in the soluble fraction (Fig. 3A), and was found to be enzymatically active (Fig. 3B).

Quantitation of total expressed enzyme after 4h of induction gave a value of about 10mg/liter of culture. In addition, it was found that by switching the growth medium to LB instead of M9, the yield of expressed enzyme could be doubled to 20mg/liter, with no change in the fraction of soluble enzyme (see following section). Other variations in conditions of expression, such as lower induction temperatures with longer times, or different host cell strains, gave consistently lower yields of expressed Fru-1,6-P_2ase (data not shown).

Purification and Properties of Expressed Fru-1,6-P_2ase. The protocol for the purification of Fru-1,6-P_2ase expressed in *E. coli* was adapted from that used to purify the rat liver form (12). After harvesting and lysing the cells as described above for the assay of

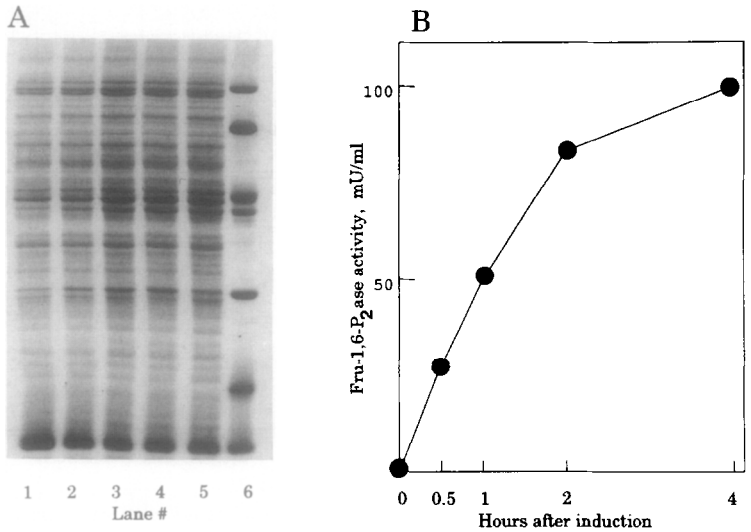
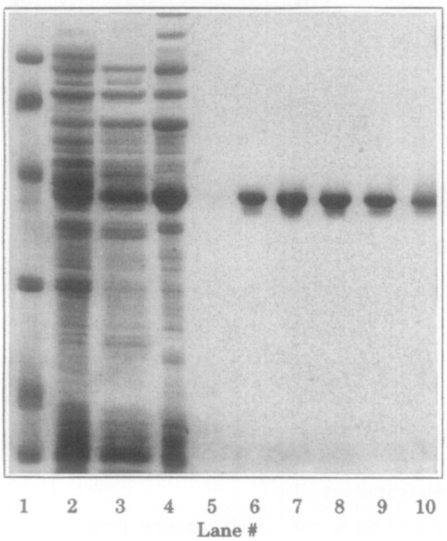


Figure 3. Fraction of Induced Fru-1,6-P_2ase that is Soluble and Active. Cell samples from the experiment shown in Fig. 2 were lysed and the lysates either subjected to SDS-PAGE or assayed for Fru-1,6-P_2ase activity as described under "Materials and Methods". A. is the Coomassie Blue-stained gel of zero h (lane 1), 0.5h (lane 2), 1h (lane 3), 2h (lane 4), and 4h (lane 5) samples after IPTG induction; lane 6, the marker standards of Fig. 2 and 2μg of purified rat liver Fru-1,6-P_2ase. B. is the Fru-1,6-P2ase activity profile.

activity, the lysate was incubated at 4°C with 2mg DNase I and 5mM $MgSO_4$ for 1h and centrifuged (15,000 x g x 20min). The supernate was adjusted to 10mM in EDTA, heated to 58°C for 3min, cooled to 4°C and the denatured protein removed by another centrifugation. Protein that precipitated between 30 and 50% saturation of $(NH_4)_2SO_4$ contained most of the Fru-1,6-P_2ase activity and was collected from the supernate by centrifugation as described above. The precipitate was dissolved in 20ml of buffer (5mM malonate, pH 5.8, 0.1mM EDTA, 1mM DTT, 0.5mM PMSF and 2.5μg/ml leupeptin) and the residual $(NH_4)_2SO_4$ removed by passage through a Sephadex G50 (medium) column equilibrated with the same buffer. The desalted sample was then applied to a 50ml column of carboxymethyl Sephadex C50, equilibrated with the same buffer, and the unbound protein was washed off to an absorbance at 280_{nm} of <0.05. Fru-1,6-P_2ase activity was then eluted by adding 2mM Fru-1,6-P_2/2mM AMP to the washing buffer. The substrate-eluted Fru-1,6-P_2ase was at least 99% pure on SDS-PAGE, and comigrated with purified rat liver Fru-1,6-P_2ase with a subunit M_r of 40,000 (Fig. 4A). A summary of the purification procedure and the yield at each step is shown in Fig. 4B. The final yield from three liters of bacterial culture was approximately 17mg or slightly less than 6mg/liter.

A comparison of the kinetic properties of the *E.coli* expressed enzyme with those of enzyme purified from rat liver shows them to have the same specific activity, K_m for Fru 1,6-P_2, and K_i for AMP and for Fru 2,6-P_2 (Table I).

A



B

Summary of Purification of *E.coli* Expressed Fru-1,6-P_2ase

Fraction	Volume (ml)	Units	Protein (mg)	S.A U/mg	Yield (%)
Lysate supernate	185	814	610	1.3	100
Heat step supernate	185	650	277	2.6	88
Sephadex G50 eluate	41	586	56	10.5	72
CM-Sephadex eluate	2	300	17	17.8	37

Figure 4. Purification of Fru-1,6-P_2ase Expressed in *E.coli*. Three liters of BL(21DE3) cells containing pFBPET were grown in LB medium, induced with IPTG, and after 4h the cells were harvested and the enzyme purified from the lysate, as described under "Materials and Methods". A. Lane 1, markers standards as in Fig. 2; lane 2, lysate supernate; lane 3, heat step supernate; lane 4, Sephadex G50 eluate; lanes 5 through 9, fractions eluted from the CM-Sephadex column; and lane 10, purified rat liver Fru-1,6-P_2ase. B. Summary of purification, yields, and specific activity (S.A). The CM-Sephadex eluate, total volume 41ml, was assayed after concentration to 2ml by $(NH_4)_2SO_4$ precipitation.

Amino acid sequencing of the purified expressed Fru-1,6-P_2ase gave the following sequence from the first 10 cycles of the sequenator; Met-Val-Asp-His-Ala-Pro-Phe-Glu-Thr-Asp-, which, without the initiation Met, is the same as that obtained from sequencing the purified enzyme from rat liver (8).

DISCUSSION

This is the first report of expression of a mammalian Fru-1,6-P_2ase in a heterologous system. The expression of rat liver Fru-1,6-P_2ase in *E. coli* using a T7 RNA polymerase-transcribed plasmid vector allows for the synthesis of large amounts of enzymatically active product (~10mg/liter). The expressed enzyme was identical to the native rat liver form with respect to amino terminal sequence, subunit molecular weight, and kinetic properties. Both enzymes were inhibited by Fru 2,6-P_2 and by AMP with K_i's around 4μM and 40μM, respectively. In addition, a rapid purification procedure is described that can be completed in one day, from which homogeneous Fru-1,6-P_2ase is obtained that is suitable for crystallization.

The time-dependent appearance of Fru-1,6-P_2ase activity paralleled that for total enzyme production, which suggests that proper folding of the enzyme was simultaneous with, or closely followed, its synthesis. The greatest amount of expressed soluble Fru-1,6-P_2ase

TABLE I

Comparison of Physical and Kinetic Properties of Purified *E.coli* Expressed and Rat Liver fructose-1,6-bisphosphatases

Property	pFBPET	RLFBP*
Subunit M_r	40,000	40,000
Specific activity	17.8U/mg	17.6U/mg
K_m	~200nM	~200nM
K_i, Fru 2,6-P_2	4μM	3.3μM
K_i, AMP	40μM	30μM

* RLFBP, rat liver fructose-1,6-bisphosphatase.

was obtained when the rates of induction were highest, such as at 37°C and in rich media, which may be due to the relative thermal stability of Fru-1,6-P_2ase (Fig. 4B and 12). This is in contrast to the formation in *E.coli* of soluble forms of other enzymes of intermediary metabolism, such as glycogen phosphorylase (15), glucokinase (14) and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (16), whose recovery was enhanced by lowering the induction temperature to 22°C, and whose activity is labile *in vitro* at 37°C (16).

Substitution of the bacterial host cell by a pLysS plasmid-containing *E.coli* strain which encodes lysozyme (10), reduced the recovered Fru-1,6-P_2ase by 50% (data not shown). Lysozyme inhibits T7 RNA polymerase (10), and, as such, decreases the rates of transcription of pET vectors, a property that enhanced the yield of soluble glucokinase and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (16) but not of Fru-1,6-P_2ase.

Inducing the expression of large amounts of Fru-1,6-P_2ase in the host cell, which already contains a high level of 6-phosphofructo-1-kinase, creates a futile substrate cycle at the expense of ATP, and may be the reason for the greatly diminished rates of protein synthesis seen after 2h (Fig. 3B).

The mechanism of inhibition of liver Fru-1,6-P_2ase by Fru 2,6-P_2 has been controversial; Fru 2,6-P_2 has been postulated to bind at the substrate site (3), or to a separate allosteric site (4), or to bind at both substrate and allosteric sites (5). Recent X-ray crystallographic analyses of the pig kidney enzyme done in the presence of effectors support the competitive inhibition hypothesis (7). However, definitive identification of the residues responsible for substrate and/or effector binding can only be accomplished by the expression of sufficient amounts of native and mutant enzyme forms. For example, Lys-274 of pig kidney Fru-1,6-P_2ase has been postulated to interact with the 2- and 6-phosphate groups and the 5-oxygen atom of Fru 2,6-P_2 (7), and may also be a key to the mechanism of hydrolysis. The expression system reported here will allow characterization of site-specific mutants of this and other postulated effector sites.

REFERENCES

1. Claus, T.H., El-Maghrabi, M.R., Regen, D.M., Stewart, B., McGrane, M., Kountz, P., Nyfeler, F., Pilkis, J., & Pilkis, S.J. (1984) *Curr. Top. Cell. Regul.* 23, 57-86.

2. Hers, H.G., & Hue, L. (1983) *Annu. Rev. Biochem.* 52, 617-653.

3. Pilkis, S.J., El-Maghrabi, M.R., McGrane, M., Pilkis, J., & Claus, T.H. (1981) *J. Biol. Chem.* 256, 11489-11495.

4. François, J., Van Schaftingen, E., & Hers., H.G. (1983) *Eur. J. Biochem.* 134, 269-273.

5. Meek, D.W., & Nimmo, H.G. (1984) *Biochem. J.* 222, 131-138.

6. Marcus, F., Edelstein, I., Reardon, I., & Heinrikson, R.L. (1982) *Proc. Natl. Acad. Sci. USA* 79, 7161-7165.

7. Ke, H., Thorpe, C.M., Seaton, B.A., Lipscomb, W.N., & Marcus, F. (1989) *J. Miol. Biol.* 212, 513-539.

8. El-Maghrabi, M.R., Pilkis, J., Marker, A., Colosia, A.D., D'Angelo, G., Fraser, B., & Pilkis, S.J. (1988) *Proc. Natl. Acad. Sci. USA* 85, 8430-8434.

9. Studier, F.W., & Moffat, B.M. (1986) *J. Miol. Biol.* 189, 113-130.

10. Studier, F.W., Rosenberg, A.H., Dunn, J.J., & Dubendorff, J.W. (1990) *Methods Enzymol.* 185, 60-89.

11. Laemmli, U.K. (1970) *Nature* 227, 680-685.

12. Riou, J.P., Claus, T.H., Flockhart, D.A., Corbin, J.D., and Pilkis, S.J. (1977) *Proc. Natl. Acad. Sci. USA* 74, 4615-4619.

13. Lowry, O.H., Rosebrough, N.J., Farr, A.L., & Randall, R.J. (1951) *J. Biol. Chem.* 193, 265-275.

14. Chen, C.T., Tauler, A., Lange, A.J., Chan, K., Printz, R.L., El-Maghrabi, M.R., Granner, D.K., & Pilkis, S.J. (1989) *Biochem. Biophys. Res. Commun.* 165, 817-825.

15. Browner, M.F., Rosen, P., Tugendreich, S., & Fletterick, R. (1991) *Protein Engineering* in press.

16. Lin, K., Kurland, I., Xu, L.Z., Lange, A.J., Pilkis, J., El-Maghrabi, M.R., & Pilkis, S.J. (1991) *Protein Expression* in press.