BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **250,** 531–535 (1998)
ARTICLE NO. RC989342

Use of the Glyceraldehyde-3-phosphate Dehydrogenase Promoter for Production of Functional Mammalian Membrane Transport Proteins in the Yeast *Pichia pastoris*

Frank Döring, Maja Klapper, Stephan Theis, and Hannelore Daniel[1]
Institute of Nutritional Sciences, University of Giessen, Wilhelmstrasse 20, D-35392 Giessen, Germany

Received August 14, 1998

The promoter of the glyceraldehyde-3-phosphate dehydrogenase gene (P_{GAP}) was employed to produce the mammalian peptide transporters hPEPT1 and rPEPT2 as models for polytopic transmembrane proteins in the methylotrophic yeast *Prichia pastoris.* Cells of a recombinant renal peptide transporter (rPEPT2) clone produced constitutively the functional carrier protein. The level of functional expression of rPEPT2 with P_{GAP} varied depending on the carbon source used for cell growth, but was up to five times higher than that obtained with the commonly employed inducible alcohol oxidase 1 promoter (P_{AOX1}). Similar results were obtained for the expression level of the human intestinal peptide transporter hPEPT1 controlled by either P_{GAP} or P_{AOX1}. Therefore, the P_{GAP} seems to be an attractive alternative to P_{AOX1} for generation of transgenic *P. pastoris* cells expressing functional mammalian membrane transport proteins at high levels.

Key Words: **constitutive expression; polytopic membrane proteins; peptide transporters; methylotrophic yeast.**

Although the methylotrophic yeast *P. pastoris* has been used frequently as a host for high level expression of foreign proteins (1, 2) the number of membrane proteins succesfully expressed in yeast is very low. Notable exceptions are some mammalian G-protein coupled receptors and di-/tripeptide transporters (3–8). All these polytopic membrane proteins were functionally produced in *P. pastoris* by using the methanol-inducible promoter of the alcohol oxidase 1 gene (P_{AOX1}). Since the level of expression by the P_{AOX1} depends on culture conditions, i.e. methanol concentrations, induction time and methanol utilizing phenotype (9), we tested the recently isolated promoter (10) of the glyceraldehyde-3-phosphate dehydrogenase gene (P_{GAP}) for constitutive production of the mammalian peptide transporters hPEPT1 and rPEPT2 as models for mammalian cell membrane carrier proteins.

hPEPT1 and rPEPT2 are members of the PTR family of proton dependent oligopeptide transporters (11). The carrier proteins mediate electrogenic pH-gradient dependent transmembrane transport of di-/tripeptides and selected peptidomimetics including β-lactam antibiotics in a variety of tissues (12). By this the transporters contribute to amino acid nitrogen handling and affect absorption, elimination and pharmacokinetics of xenobiotics. rPEPT2 represents a 729 amino acid protein with 12 putative transmembrane domains, 5 potential N-glycosylation sites and several canonical protein kinase C recognition regions. The carrier protein has been characterized after expression in *Xenopus laevis* oocytes (13), HeLa (14) and LLC-PK_1 cells (15). More recently, we characterized rPEPT2 after expression in *P. pastoris* by using the P_{AOX1} (8). hPEPT1, mainly expressed in intestine, has a comparable structure, consists of 708 amino acids, and represents in contrast to rPEPT2 a low affinity phenotype transporter (16).

The aims of this study was (a) to construct *P. pastoris* strains which constitutively produce the functional hPEPT1 and rPEPT2 proteins and (b) to compare the level of functional production when expressed either under the control of P_{GAP} or P_{AOX1}. To our knowledge, this is the first example of constitutive heterologous production of integral membrane proteins in *P. pastoris*.

[1] To whom correspondence should be addressed at Institute of Nutritional Sciences, Justus-Liebig-University Giessen, Wilhelmstrasse 20, 35392 Giessen, Germany. Fax: +641 99 39049. E-mail: hannelore.daniel@ernaehrung.uni-giessen.de.

Abbreviations used: P_{GAP}, promoter of the GAPDH gene from *P. pastoris;* GAPDH, glyceraldehyde-3-phosphate dehydrogenase; P_{AOX1}, promoter of the alcohol oxidase 1 gene; rPEPT2, rabbit renal peptide transporter; hPEPT1, human intestinal peptide transporter; OD, optical density 600 nm (one OD equals approximately 5×10^7 cells), PTR, peptide transporter.

0006-291X/98 $25.00

MATERIALS AND METHODS

Chemicals, E. coli strain, and P. pastoris strains. Chemicals were obtained from Sigma (Deisenhofen, Germany). Custom-synthesized ^{3}H-(D)-phenylalanine-(L)-alanine (^{3}H-D-Phe-Ala) with a specific activity of 22.5 Ci/mmol was purchased from Zeneca (Billingham, UK). *E. coli* strain XL-1 Blue (Stratagene, Heidelberg, Germany) was used for transformation and propagation of the recombinant plasmids. The *P. pastoris* strain GS115 (Invitrogen, San Diego, USA) deficient in histidinol dehydrogenase activity (*his4*) was used in the expression study.

Transformation and culture of P. pastoris. Transformations of yeast cells were performed according to the lithium chloride method as described in the manual of Version A of the expression plasmid pGAPZB (Invitrogen, San Diego, USA). *P. pastoris* strains were cultured as described in the manual Version E of the *Pichia* expression kit (Invitrogen, San Diego, USA). The following media were used: YPD, 1 % yeast extract, 2 % peptone, 2 % glucose; MDH, 1.34% yeast nitrogen base without amino acid (YNB), 4×10^{-5} % biotin, 0.004 % histidine, 1 % glucose; MGYH, 1.34 % YNB, 4×10^{-5} % biotin, 0.004 % histidine, 1 % glycerol; MMH, 1.34 % YNB, 4×10^{-5} % biotin, 0.004 % histidine, 1 % methanol). Cell density in liquid cultures was monitored by measuring optical density at 600 nm.

Cloning procedures and construction of P. pastoris strains. Cloning procedures were essentially performed as described (17). The expression plasmid containing the P_{GAP} was obtained from Invitrogen (Version A; San Diego, USA). The plasmid pGAPZB-rPEPT2 was constructed as described below (3.1). The characteristic restriction fragments obtained from the pGAPZB-rPEPT2 digest (*Avr*ll, 7149 bp; *Eco*RI, 3278 bp and 3871 bp) confirmed that the plasmid contains the rPEPT2 cDNA.

For insertion of the hPEPT1 into the expression vectors pPICZB and pGAPZB (Invitrogen, San Diego, USA) containing P_{AOX} resp. P_{GAP}, two restriction sites were introduced in the cDNA of hPEPT1 by PCR-mediated mutagenesis. The hPEPT1 cDNA was amplified from pBluescript SK$^-$-hPEPT1 (16) using PCR primers with the desired restriction sites as 5′ extensions (f1: 5′-GTCG-CAGGAGCCC**TTCGAA**CCGCCGCCAT G GGAATGTCC-3′, *Sfu*I site; b1: 5′-CTTGCCTCCT**TCTAGA**CACATCTGTTTCTGTGAAT TGGCCCC-3′, *Xba*I site). The 2.2 kb PCR fragment was first inserted into the pCRII (Invitrogen, San Diego, USA) cloning vector. hPEPT1 was then inserted into the *Sfu*I/*Xba*I sites of the vectors pPICZB and pGAPZB. The resulting vectors pPICZB-hPEPT1 and pGAPZB-hPEPT1 were linearized with *Avr*II and used to transform the *P. pastoris* strain GS115. The obtained transformants were named GS-hPEPT1 (transformed with pPICZB-hPEPT1) respectively GAP-hP1 (transformed with pGAPZB-hPEPT1). Cells of ten transformants were tested for uptake of ^{3}H-D-Phe-Ala in comparison to isogenic control strains. Clones displaying the highest uptake rates were used for further studies.

Expression of rPEPT2 and hPEPT1 in different P. pastoris strains and peptide uptake assay. Cells of the clones GAP-rP2, GAP-hP1 and GAP-con (control) were grown to an OD of 1-2 units/ml in YPD, MDH, MGYH, or MMH medium and were used for uptake measurements. To induce the expression of rPEPT2 and hPEPT1 in the strains GS-RPEPT2 (8) and PICZ-hP1 (this study), cells were grown first in MGYH medium and than for 24 h in minimal medium containing methanol (MMH). Uptake measurements were performed at 22–24°C by using a rapid filtration technique with Schleicher & Schuell filters (RC 55 type, 0.45 μm pore size) as described previously (7). Cells were harvested by centrifugation (10 min) at 3000 $\times$ g, washed once with 100 mM potassium phosphate buffer and resuspended to 1 OD_{600nm} units/10 μl in buffer. Uptake was initiated by rapidly mixing 10 μl cells with 40 μl 100 mM phosphate buffer containing ^{3}H-D-Phe-Ala. After 30 min, incubation of cells was terminated by addition of ice cold buffer followed by filtration. The filter was washed twice and radioactivity associated with the filter was determined.

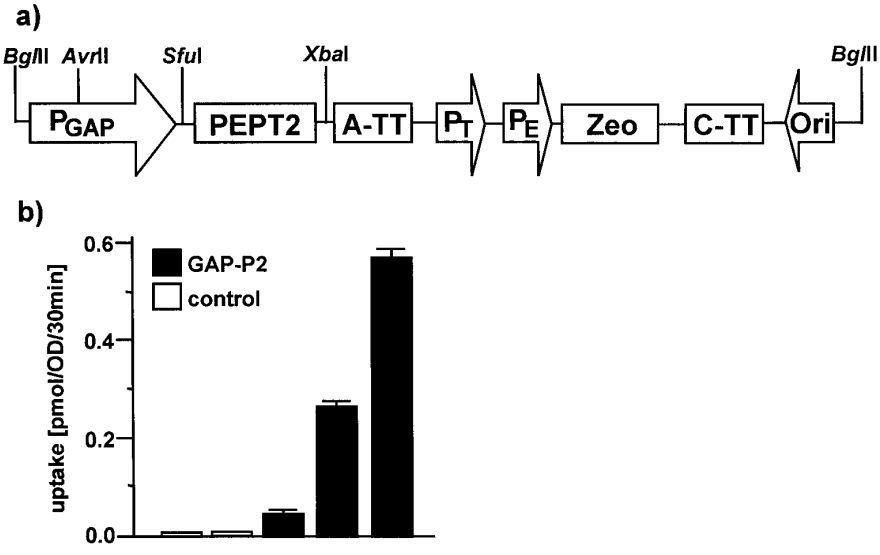

FIG. 1. (a) Map of rPEPT2 expression plasmid pGAPZB-rPEPT2, P_{GAP}, *P. pastoris* GAPDH promoter; rPEPT2, cDNA of rPEPT2; A-TT, AOX1 transcription termination region; P_T, *S. cerevisiae* transcription elongation factor 1 promoter that drives expression of the Sh ble gene in *P. pastoris;* P_E, synthetic constitutive promoter that drives expression of the Sh ble gene in *E. coli;* Zeo, *Streptoalloteichus hindustanus* bleomycin gene (Sh ble) or Zeocin resistance gene; C-TT, CYC1 transcription termination region; Ori, origin of DNA replication in *E. coli;* *Avr*II, *Bg*/II, *Sfu*I, *Xba*I, positions of the relevant restriction enzymes. (b) Uptake of ^{3}H-D-Phe-Ala into the *P. pastoris* transformed with pGAPZB-rPEPT2 or the parent vector pGAPZB. *P. pastoris* GS115 was transformed with 10 μg *Avr*II-linearized plasmid pGAPZB-rPEPT2 or pGAPZB. Transformants were selected by growth on YPD agar plates supplemented with Zeocin (100 μg/ml). Transport of ^{3}H-D-Phe-Ala (2 μCi/ml; 0.09 μM) into the cells of the clones GAP-rP2 (transformed with pGAPZB-rPEPT2) and GAP-Con (transformed with pGAPZB) was measured for 30 min of incubation at pH 7.0.

RESULTS AND DISCUSSION

Construction of rPEPT2 expression vector and rPEPT2-expressing P. pastoris strain. A *Sfu*I/*Xba*I 2764 bp cDNA fragment encoding the peptide transporter rPEPT2 from the plasmid pPIC3L-rPEPT2 (8) was subcloned into the *Sfu*I/*Xba*I sites of the expression plasmid pGAPZB. In the resulting vector pGAPZB-rPEPT2 (Fig. 1a) the rPEPT2 cDNA was under the transcriptional control of P_{GAP}. The plasmid pGAPZB-rPEPT2 and the parent vector pGAPZB were linearized with *Avr*II and used to transform the *P. pastoris* host strain GS115. Three of the transformants were tested for functional expression of rPEPT2. For this purpose, cells of recombinant clones were grown in glucose containing medium (YPD) and uptake of the dipeptide ^{3}H-D-Phe-Ala was measured. As shown in Fig. 1b, the rPEPT2 clones tested showed up to 140 times higher peptide influx rates than the isogenic control clone. Subsequently, the rPEPT2 clone (GAP-rP2) with the highest uptake rate was used for further studies.

Transport characteristics of rPEPT2 when constitutively expressed in P. pastoris. Uptake of ^{3}H-D-Phe-Ala into the selected rPEPT2-clone as a function of incubation time was determined for periods of 1 to 180 min. As shown in Fig. 2a, uptake of ^{3}H-D-Phe-Ala into rPEPT2-expressing cells was linear for up to 40 min with a mean influx rate of 0.81 ± 0.02 pmol/OD/min, whereas there was essentially no uptake in the control cells. Based on linearity of uptake with respect to time, an incubation time of 30 min was chosen for all subsequent transport studies.

To demonstrate the pH dependence of rPEPT2-mediated transport of D-Phe-Ala into *P. pastoris,* the extracellular pH was changed to cover a pH range to 5.0 to 9.0. As shown in Fig. 2b, uptake of D-Phe-Ala as a function of medium pH into *P. pastoris* expressing rPEPT2 displays a pronounced pH dependence with a pH optimum of 6.5 to 7.0. Lowering pH from 7.0 to 5.0 caused a decrease in rPEPT2 activity by 30 % when compared with the peak uptake at pH 7.0. A similar pH dependence of rPEPT2-mediated transport function was found for example in native renal brush border membrane vesicles (18). ^{3}H-D-Phe-Ala influx as a function of substrate concentration into rPEPT2-expressing GAP-rP2 cells displayed Michaelis-Menten-type saturation kinetics (Fig. 2c). The obtained app. $K_{0.5}$ value was 0.122 mM ± 0.012 mM (Fig. 2d), that is almost identical to that obtained in other expression systems (18, 13). We therefore conclude that the key characteristics of this peptide transporter phenotype are conserved in GAP-rP2 cells.

Level of functional production of rPEPT2 and hPEPT1. As shown in Table 1, the maximal transport activity (app. V_{max}) of rPEPT2 in the strain GAP-rP2

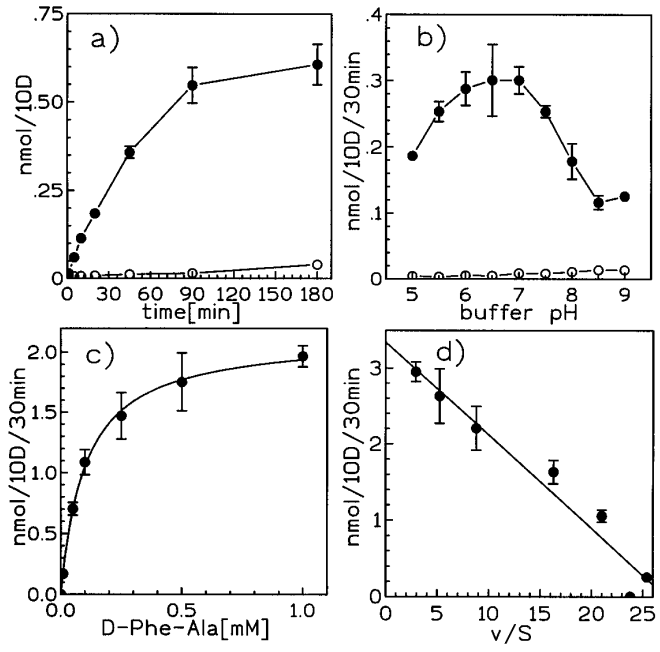

FIG. 2. Transport characteristics of rPEPT2 constitutively expressed in *P. pastoris.* Uptake of ^{3}H-D-Phe-Ala into *P. pastoris* expressing rPEPT2 as function of incubation time (a), extracellular pH (b) and substrate concentration (c). Influx of ^{3}H-D-Phe-Ala (2 μCi/ml) into the cells of the rPEPT2 expressing strain GAP-rP2 (●) and the isogenic control strain GAP-Con (○) was measured for 30 min of incubation at pH 7.0 (a,c) or pH 5.0 to 9.0 (b) in the presence of 5 μM (a,b) or increasing concentrations (C, 0.001 to 1 mM) of unlabeled substrate. (c) Uptake rates in control cells were subtracted from the corresponding uptake rates in rPEPT2 expressing cells. The resulting net transport rates were transformed according to Eadie-Hofstee (d) and app. kinetic constants were derived by linear regression analysis by the least-squares method.

depended on the carbon source used for cell growth. In contrats to the maximal transport capacity substrate affinity remained essentially unaffected. When GAP-rP2 cells were grown in a mimimal medium containing glucose, app. V_{max} of rPEPT2 was approximately 2 or 8 times higher than in cells grown in glycerol- or methanol containing medium. Similar differences in the level of production depending on the carbon source used were recently obtained by P_{GAP}-promoter studies employing the soluble enzyme beta lactamase as a reporter (10). Taken together, the constitutively expressed *P. pastoris* P_{GAP} is strongly regulated by the carbon source, a phenomenon well documentated in other yeast species such as *S. cerevisiae* (19).

The level of functional production of rPEPT2 under control of P_{GAP} in cells grown in glucose was 3 (minimal medium) to 5 (complex medium) times higher than in cells expressing rPEPT2 under control of P_{AOX1} (Table 1). Although it is not known whether this is caused by different rates of protein synthesis and/or by different efficiencies of vesicle-mediated transport of synthetized protein to the cell membrane, the increased maximal transport capacity per cell and thus the increased

TABLE 1

Kinetic Characteristics of rPEPT2 and hPEPT1 with Their cDNAs Expressed under the Control of P_{GAP} or P_{AOX1}

Kinetic constants	YPD-gluc	Glucose	Glycerol	Methanol	P_{AOX1}-rPEPT2 Methanol
		P_{GAP}-rPEPT2			
$K_{0.5}$ [μM]	122 ± 12	186 ± 43	134 ± 14	110 ± 13	168 ± 12
V_{max} [nmol/OD/30min]	3.34 ± .020	2.06 ± 0.17	1.11 ± 0.07	0.41 ± 0.02	0.60 ± 0.03
		P_{GAP}-hPEPT1			P_{AOX1}-hPEPT1
$K_{0.5}$ [mM]	1.26 ± 0.16	1.49 ± 0,18	1.12 ± 0.25	1.41 ± 0.45	1.09 ± 0.17
V_{max} [nmol/OD/30min]	5.65 ± 0.19	3.16 ± 0.18	2.56 ± 0.16	0.21 ± 0.02	0.65 ± 0.07

Note. Cells of the recombinant clones GAP-rP2 and GAP-hP1 expressing the cDNAs of either rPEPT2 or hPEPT1 under the control of P_{GAP} (P_{GAP}-rPEPT2, P_{GAP}-hPEPT1) were grown in complex medium containing glucose (YPD-gluc) or in minimal medium containing glucose (MDH), glycerol (MGYH), or methanol (MMH). The construction of the strains GS-rPEPT2 and GS-hPEPT1 expressing the cDNAs of rPEPT2 or hPEPT1 under the control of P_{AOX1} (P_{AOX1}-rPEPT2, P_{AOX1}-hPEPT1) has been described previously (8) or is provided in the methods section. Influx of ^{3}H-D-Phe-Ala (2 μCi/ml) into the *P. pastoris* cells was measured for 30 min of incubation at pH 7.0 in the presence of increasing concentrations of unlabeled substrate (0.0001 to 10 mM). Uptake rates in control cells were subtracted from the corresponding uptake rates in rPEPT2 or hPEPT1 expressing cells. The resulting net transport rates were transformed according to Eadie-Hofstee and app. kinetics constants ($K_{0.5}$, V_{max}) were derived by linear regression analysis by the least-squares method.

number of functional protein will allow a more efficient purification of the recombinant protein in quantities necessary for biophysical studies.

To prove that the P_{GAP} may in general provide a suitable promoter for the expression of mammalian transport proteins, we additionally expressed the human intestinal peptide transporter hPEPT1 by using P_{GAP}. Cells of the constructed *P. pastoris* strain (GAP-hP1) expressed the functional carrier protein with its phenotypical characteristics such as pH-dependence, substrate specificity and low substrate affinity (not shown). When compared with cells grown under control of P_{AOX1} apparent V_{max} in P_{GAP}-controlled cells was again 5 times higher (Table 1). This shows that hPEPT1 expression rate like that of rPEPT2 is significantly increased and suggests that P_{GAP} is superior in driving production of mammalian transmembrane proteins in *P. pastoris*.

CONCLUSIONS

(1) By employing the P_{GAP} we constructed *P. pastoris* strains producing the mammalian proton coupled peptide transporters rPEPT2 and hPEPT1. The characteristic transport properties of the high and low affinity type transporter were conserved in the transgenic yeast cells.

(2) The level of functional production of both carrier proteins under control of P_{GAP} in cells grown in glucose medium was up to five times higher than that of the commonly used P_{AOX1} in cells grown in methanol medium. Therefore, P_{GAP} seems to be the promoter of choice for heterologous production of polytopic membrane proteins in *P. pastoris*.

ACKNOWLEDGMENTS

We thank M. A. Hediger for providing the cDNA of hPEPT1. This work was supported by the Deutsche Forschungsgemeinschaft (Da 190/5-1) to H. Daniel.

REFERENCES

1. Cregg, J. M., Vedvick, T. S., and Raschke, W. C. (1993) *Bio/Technology* **11,** 905–910.
2. Hollenberg, C. P., and Gellissen, G. (1997) *Curr. Opin. Biotechnol.* **8,** 554–560.
3. Weiss, H. M., Haase, W., Michel, H., and Reilander, H. (1995) *FEBS Lett.* **377,** 451–456.
4. Weiss, H. M., Haase, W., Michel, H., and Reilander, H. (1998) *Biochem. J.* **330,** 1137–1147.
5. Talmont, F., Sidobre, S., Demange, P., Milon, A., and Emorine, L. J. (1996) *FEBS Lett.* **394,** 268–272.
6. Abdulaev, N. G., Popp, M. P., Smith, W. C., and Ridge, K. D. (1997) *Protein Expr. Purif.* **10,** 61–69.
7. Döring, F., Theis, S., and Daniel, H. (1997) *Biochem. Bioph. Res. Com.* **232,** 656–662.
8. Döring, F., Michel, T., Rösel, A., Nickolaus, M., and Daniel, H. (1998) *Mol. Membr. Biol.* **15,** 79–88.
9. Screekrishna, K., Brankamp, R. G., Kropp, K. E., Blankenship, D. T., Tsay, J. T., Smith, P. L., Wierschke, J. D., Subramaniam, A., and Birkenberger, L. A. (1997) *Gene* **190,** 55–62.
10. Waterham, H. R., Digan, M. E., Koutz, P. J., Lair, S. V., and Cregg, J. M. (1997) *Gene* **186,** 37–44.
11. Steiner, H.-Y., Naider, F., & Becker, J. M. (1995) *Mol. Microbiol.* **16,** 825–834.
12. Leibach, F. H., and Ganapathy, V. (1996) *Annu. Rev. Nutr.* **16,** 99–119.
13. Boll, M., Herget, M., Wagener, M., Weber, W.-M., Markovich, D., Biber, J., Clauss, W., Murer, H., and Daniel, H. (1996) *Proc. Nat. Acad. Sci. USA* **93,** 284–289.
14. Liu, W., Liang, R., Ramamoorthy, S., Fei, Y.-J., Ganapathy,

M. E., Hediger, M. A., Ganapathy, V., and Leibach, F. H. (1995) *Biochem. Biophys. Acta* **1235,** 461–466.

15. Saito, H., Okuda, M., Terada, T., Sasaki, S., and Inui, K. I. (1995) *J. Pharmacol. Exp. Ther.* **275,** 1631–1637.
16. Liang, R., Fei, Y.-J., Prasad, P. D., Ramamoorthy, S., Han, H., Yang-Feng, T. L., Hediger, M. A., Ganapathy, V., and Leibach, F. H. (1995) *J. Biol. Chem.* **270,** 6456–6463.
17. Sambrook, J., Fritsch, E. F., and Manniatis, T. L. (1989) Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
18. Daniel, H., Morse, E. L., and Adibi, S. A. (1991) *J. Biol. Chem.* **266,** 19917–19924.
19. Kuroda, S., Otaka, S., and Fujisawa, Y. (1994) *J. Biol. Chem.* **269,** 6153–6162.