Biochemical and Biophysical Research Communications **267,** 169–173 (2000)
doi:10.1006/bbrc.1999.1946, available online at http://www.idealibrary.com on IDEAL®

Expression of Functional Soluble Forms of Human β-1,4-Galactosyltransferase I, α-2,6-Sialyltransferase, and α-1,3-Fucosyltransferase VI in the Methylotrophic Yeast *Pichia pastoris*

M. Malissard,[1] S. Zeng, and E. G. Berger
Institute of Physiology, University of Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland

Received November 19, 1999

The cDNAs encoding soluble forms of human β-1,4-galactosyltransferase I (EC 2.4.1.22), α-2,6-sialyltransferase (EC 2.4.99.1), and α-1,3-fucosyltransferase VI (EC 2.4.1.65), respectively, have been expressed in the methylotrophic yeast *Pichia pastoris.* The vector pPIC9 was used, which contains the N-terminal signal sequence of *Saccharomyces cerevisiae* α-factor to allow entry into the secretory pathway. The recombinant enzymes had similar kinetic properties as their native counterparts. Their identity was confirmed by Western blotting. Recombinant enzymes may be used for *in vitro* synthesis of oligosaccharides. © 2000 Academic Press

***Key Words:* β-1,4-galactosyltransferase I; α-2,6-sialyltransferase; α-1,3-fucosyltransferase VI; heterologous expression; yeast.**

Heteroglycans are macromolecules which exert multiple biospecific functions by virtue of their highly ordered structures (1, 2). The difficulties in obtaining sufficient quantities for investigation of their biological function are notorious. Although enormous progress has been made in the chemical synthesis of heterooligosaccharides (3), their synthesis at the gram scale still appears beyond any reasonable cost-benefit ratio. As an alternative to chemical synthesis, chemoenzymatic or even enzymatic synthesis is now within the grasp of possibilities. The use of glycosyltransferases as tools for oligosaccharides synthesis is very attractive because of their regioselectivity and stereospecificity (4) but their application as industrial catalysts still awaits their availability in mass quantities. Several reports on successfull heterologous expression in *E. coli* (5, 6), yeast (for a review see 7) and in higher eukaryotic cells appeared (8–10). However, these enzymes were not expressed at an industrial scale.

Yeasts as host cells are considered safe, cheap and amenable to high expression levels; therefore, they are good candidates for production of recombinant glycosyltransferases at industrial scale.

In the past we used the BT150 strain of *S. cerevisiae* to express human β-1,4-galactosyltransferase I (gal-T I), α-2,6-sialyltransferase (ST6Gal), and α-1,3-fucosyltransferase VI (fucT VI). These proteins were expressed both as full length forms and soluble proteins lacking the transmembrane domain. Expression levels for soluble forms were 0.22, 0.001, and 0.001 U/liter for gal-T I, ST6Gal, and fucT VI, respectively. Further optimization of fermentation conditions of recombinant gal-T I (rgal-T I) and recombinant N-deglycosylated gal-T I (rNdgal-T I) yielded 15 U/liter and 30 U/liter, respectively (11, 12). rgal-T I has been shown to be N-hyperglycosylated (13). In fact, *S. cerevisiae* is known to synthesize polymannans which can considerably impair biological activity of the recombinant proteins. To overcome problems due to hyperglycosylation we investigated the yeast expression system *Pichia pastoris.* Until now, only 3 glycosyltransferases were expressed using the *P. pastoris* expression system (14–16).

Here we show that soluble forms of human gal-T I, ST6Gal, and fucT VI will be good candidates for scaled-up expression in the methylotrophic yeast *P. pastoris.* In this report we demonstrate the presence of recombinant gal-T I, ST6Gal, and fucT VI in the supernatant of *P. pastoris* cultures as immunoreactive enzymes having similar kinetic properties as their native counterparts.

Abbreviations used: gal-T I, β-1,4-galactosyltransferase I (EC 2.4.1.22); Ndgal-T I, *N*-deglycosylated gal-T I; ST6Gal, α-2,6-sialyltransferase (EC 2.4.99.1); fucT VI, α-1,3-fucosyltransferase VI (EC 2.4.1.65); GlcNAc, *N*-acetylglucosamine; LacNAc, *N*-acetyllactosamine.

[1] To whom correspondence should be addressed. Fax: +/41/1/635 6814. E-mail: malissar@physiol.unizh.ch.

0006-291X/00 $35.00

TABLE I

Primers Used for PCR Amplification of Gal-T I, ST6Gal, and FucT VI

Primer's name	Sequence
GT *Eco*.up	5′ CCAGAATTCTCTCGAGAAAAGAGAGGCT 3′
GT *Xho*.down	5′ AGACTGGCGTTGTAATGAGTAGTGTTCC 3′
ST *Pvu*.up	5′ TACAGCTGGAAAAGAAGAAAGGGAGT 3′
ST *Xho*.down	5′ CACTCGAGTTAGCAGTGAATGGTCC 3′
FT *Nru*.up	5′ CATCGCGAGTGTCTCAAGACGA 3′
FT *Xho*.down	5′ GTCTCGAGCTCTCAGGTGAACCAA 3′

MATERIALS AND METHODS

Media Composition

MD (1.34% Yeast Nitrogen Base, 4×10^{-5}% Biotin, 1% dextrose). BMGY (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6, 1.34% Yeast Nitrogen Base, 4×10^{-5}% Biotin, 1% glycerol). BMMY (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6, 1.34% Yeast Nitrogen Base, 4×10^{-5}% Biotin, 1% methanol).

Yeast Strains

P. pastoris GS115 (*his4*) and KM71 (*arg4his4aox1: ARG4*) were used as host strains for the expression of the glycosyltransferases. They were from Invitrogen Corp. (San Diego, CA).

Construction of Recombinant Vectors

The cDNA encoding soluble forms of human gal-T I, ST6Gal, and fucT VI were generated by PCR and subcloned into the vector pPIC9 (Invitrogen Corp., San Diego, CA) for the extracellular expression of the enzymes in *P. pastoris*.

For gal-T I, primers were designed to generate an *Eco*RI site at the 5′ end and a *Xho*I site at the 3′ end (Table I). The plasmids pDPsGT and pDPsGTN69D were used as templates (13). The purified PCR products and pPIC9 were digested with *Eco*RI and *Xho*I and ligated to give pPIC9/gal-T I or pPIC9/Ndgal-T I.

For ST6Gal, primers were designed to generate a *Pvu*II site at the 5′ end and a *Xho*I site at the 3′ end (Table I). The plasmid pDPSIA was used as template (17). The purified PCR product was digested with *Pvu*II and ligated into pPIC9 digested with *Snab*I to give pPIC9/ST6Gal.

For fucT VI, primers were designed to generate a *Nru*I site at the 5′ end and a *Xho*I site at the 3′ end (Table I). The plasmid γFT-VI/pcDNA-Amp was used as template (18). The purified PCR product was digested with *Nru*I and *Xho*I and ligated into pPIC9 digested with *Not*I and filled with Klenow fragment to give pPIC9/fucT VI.

The constructs were confirmed by DNA sequencing.

Transformation of Yeast Cells and Expression of Recombinant Glycosyltransferases in Shake Flask Cultures

Plasmids pPIC9/gal-T I, pPIC9/Ndgal-T I, pPIC9/ST6Gal, and pPIC9/fucT VI were propagated in *E. coli* strain TOP 10F′ (Invitrogen) and the recovered DNA was linearized with *Sal*I/*Bgl*II, *Sal*I/*Bgl*II, and *Sac*I, respectively. The digested DNA were used to transform *P. pastoris* strains according to the manufacturer's instructions (Invitrogen). The transformants were selected on minimal medium agar plates (MD plates).

The positive transformants were identified by direct PCR screening according to Linder *et al.* (19).

Cells grown in 500 ml of BMGY at 28°C to an OD_{600} of 30–50 were harvested, washed once with BMMY medium, resuspended in BMMY at an OD_{600} of approximately 50, and incubated at 28°C for 1 to 5 days to induce expression. The cultures were centrifuged 20 min at 5000*g* at 4°C, and then the supernatants were concentrated 20-fold by pressure dialysis using Amicon YM 30 membrane and Centriprep 30. The cell extracts were prepared according to the Invitrogen handbook. The concentrated supernatants as well as the cell extracts were tested for glycosyltransferase activities.

Purification of α-1,3-Fucosyltransferase VI

fucT VI was purified using a single step affinity chromatography on UDP-hexanolamine-Sepharose and was carried out as described by Borsig *et al.* (18).

Enzyme Assays

The enzyme assays for gal-T I, ST6Gal, and fucT VI were conducted as previously described by Malissard *et al.* (13) and Borsig *et al.* (18, 20), respectively. All assays are based on measurements of incorporation of radioactive sugars into suitable acceptor substrates. These were GlcNAc (gal-T I), LacNAc (ST6Gal), and LacNAc (fucT VI).

One unit (U) of enzyme activity corresponds to the transfer of 1 μmol of sugar from the donor to the acceptor per min at 37°C.

Various Procedures

Determination of protein concentration. The protein concentration was determined using the bicinchoninic acid reagent (Pierce Chemicals, Rockford, IL) and bovine serum albumin as a standard, according to the manufacturer's instructions.

SDS–PAGE. Proteins were separated on 10% polyacrylamide gels according to Laemmli (21) using a Mini Protean II electrophoresis cell (BioRad). Gels were stained with Coomassie blue.

pNGase F treatment. 20 μg of total protein were denatured in 0.5% SDS, 1% β-mercaptoethanol at 100°C for 10 min, then 2 U of pNGase F were added and the mixture was incubated in 50 mM sodium phosphate buffer, pH 7.5, 1% NP-40 for 1 h at 37°C.

Immunoblotting. Polyacrylamide gels were electroblotted on nitrocellulose membrane (Schleicher & Schüll) according to Towbin *et al.* (22). Nitrocellulose membranes were first incubated with anti-gal-T I serum (1:200) (23), antiserum to ST6Gal (1:200) (24) or anti-fucT VI serum (OLI antibodies 1:200) (18) followed by goat anti-rabbit–horseradish peroxidase (1:5000) and stained with ECL developing kit according to the manufacturer's instructions (Amersham).

RESULTS AND DISCUSSION

Expression of Soluble gal-T I, ST6Gal, and fucT VI

Recombinant *Pichia pastoris* strains designed as GS115-Ndgal-T I/ST6Gal/fucT VI and KM71-Ndgal-T I/ST6Gal/fucT VI were generated as described under Materials and Methods. In each case transformation of GS115 strain gave only His^+ Mut^+ transformants, indicating that the *P. pastoris* AOX1 gene was not replaced with the glycosyltransferase expression cassette. The recombinant KM71 strains in which AOX1 and AOX2 genes have purposely been deleted were clearly Mut^S. For each recombinant strains 5 clones

TABLE II

Expression Levels and Localization of Recombinant Gal-T I, Ndgal-T I, ST6Gal, and FucT VI Expressed in *P. pastoris* KM71

	Localization	Expression level U/liter
Gal-T I	Medium	0.6
Ndgal-T I	Intracellular	0.35
ST6Gal	Medium	0.3
FucT VI	Medium	3

were examined in shake flask cultures. After 24 h, 48 h, 72 h, 96 h, and 120 h, respectively, of induction with 1% methanol, aliquots of the culture were centrifuged, the supernatants were concentrated 20-fold, dialyzed against 50 mM Tris HCl, pH 7, and tested for glycosyltransferase activity. The KM71-ST6Gal and KM71-fucT VI strains produced active enzymes. The amount of activity in the culture broth reached a maximum at 48 h of cultivation, the best expression levels were 300 mU/liter and 3 U/liter for ST6Gal and fucT VI, respectively (Table II). To investigate possible retention of recombinant enzymes in the host cells, the corresponding cell extracts were prepared and tested for activity. In contrast to the KM71-ST6Gal and KM71-fucT VI strains, the KM71-NdgalT I strains contained activity. The amount of activity reached a maximum at 48 h of cultivation under induction, the expression level was 600 mU/liter (Table II). When expressed in *P. pastoris*, soluble forms of ST6Gal and fucT VI were active and secreted; in contrast, Ndgal-T I was also active but retained in the cells.

Previously it has been reported that the secretion of homologous or heterologous glycoproteins in *S. cerevisiae* is inhibited when *N*-glycosylation sites are removed (25–27), thus we expressed the soluble form of gal-T I (rgal-T I) in which the *N*-glycosylation site is present as in the native enzyme. As described previously for the Ndgal-T I, ST6Gal and fucT VI, we obtained KM71-gal-T I expressing active enzyme. The gal-T I was secreted to the culture medium at a level of 400 mU/liter, while no activity was detected in the corresponding cell lysate. Thus, in accordance with the reports mentioned above, *N*-glycosylation of gal-T I appears necessary for secretion in *P. pastoris*.

Characterization of the Secreted Products

The 20-fold concentrated supernatants of BMMY medium were analyzed on SDS–PAGE (Fig. 1A). Only rgal-T I and rST6Gal could be detected by Coomassie blue staining. After a single step purification on UDP-hexanolamine Sepharose rfucT VI could also be detected on SDS–PAGE (Fig. 1B). The three recombinant proteins were treated with pNGase F, which revealed that all three were *N*-glycosylated (Figs. 1A and 1B).

Authenticity of recombinant gal-T I, ST6Gal and fucT VI was confirmed by immunoblotting (Fig. 2) using polyclonal antisera which were characterized previously (18, 23, 24).

The enzymatic properties of the recombinant proteins were compared with those of the native enzymes (Table III). Michaelis constants for rgal-T I, ST6Gal, and fucT VI were of the same order of magnitude as those of the native enzymes.

Previously, we explored the *S. cerevisiae* expression system for glycosyltransferases (7) by expressing soluble forms of human gal-T I, ST6Gal, and fucT VI. Only expression of gal-T I reached a high level (12), whereas those of ST6Gal and fucT VI were below 1 mU/liter (our unpublished data). Purification of gal-T I from yeast cell lysate, however, revealed that the protein was *N*-hyperglycosylated (rgal-T I 200 kDa versus 55 kDa for human milk gal-T I) (13). Since human ST6Gal and fucT VI harbour 2 and 4 potential *N*-glycosylation sites, respectively, *N*-hyperglycosylation might have

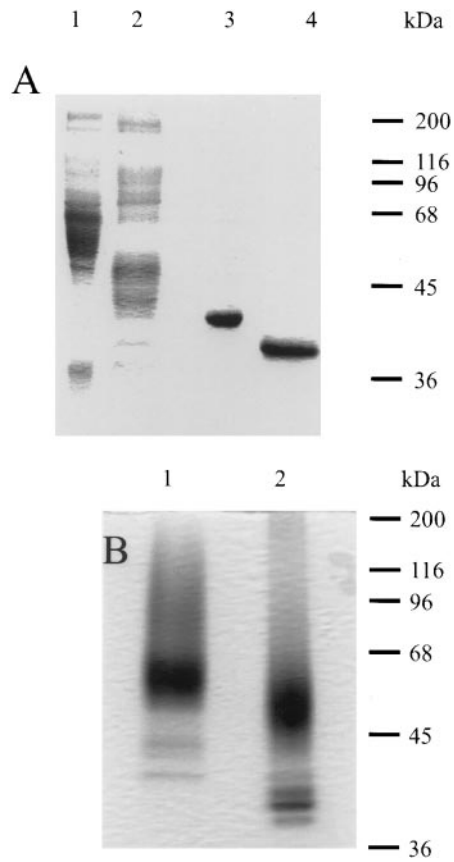

FIG. 1. SDS–PAGE analysis of rgal-T I, rST6Gal, and rfucT VI secreted in *P. pastoris* culture medium. The samples were applied on a 10% polyacrylamide gel and stained with Coomassie blue. For rgal-T I and rST6Gal we applied 20 μg of total proteins from 20-fold concentrated culture supernatant. For the purified rfucT VI we applied 5 μg of protein. (A) 1, rgal-T I; 2, rgal-T I + pNGase F; 3, rST6Gal; 4, rST6Gal + pNGase F. (B) 1, purified rfucT VI; 2, purified rfucT VI + pNGase F.

been the reason for their low enzymatic activity in *S. cerevisiae*. In order to test this hypothesis we expressed these proteins in the methylotrophic yeast *P. pastoris*, since it has been shown that the length of the oligosaccharide chains added posttranslationally to proteins in *P. pastoris* (average 8–14 mannose residues per chain) is much shorter than those in *S. cerevisiae* (50–150 mannose residues) (29). In fact, gal-T I, ST6Gal, and fucT VI expressed by *P. pastoris* are all *N*-glycosylated and secreted into the culture medium as active and immunoreactive proteins (Fig. 2). There was no evidence of hyperglycosylation as previously observed for the gal-T I expressed in *S. cerevisiae*. Thus these results suggest that hyperglycosylation of recombinant ST6Gal and fucT VI impaired their biological activity when expressed in *S. cerevisiae*. *N*-glycosylation per se, however, appears to be required in *P. pastoris* for efficient secretion into the culture medium as shown in the case of gal-T I.

Our results also infer that the choice of the signal sequence critically determines the fate of the recombinant protein. When the three glycosyltransferases were expressed in *S. cerevisiae* using the invertase signal sequence they remained associated with the cells; by contrast, the same proteins expressed in *P. pastoris* using the α-factor signal sequence were secreted into the culture medium. In fact, the α-factor from *S. cerevisiae* appeared to be more efficient than the invertase signal sequence, as reported by Schwientek and Ernst (30), who showed that a soluble form of gal-T I fused to the α-factor was secreted into the culture supernatant when expressed in *S. cerevisiae*.

In conclusion, using the yeast expression system *P. pastoris*, we were able to produce active soluble recombinant gal-T I, ST6Gal, and fucT VI which display properties similar to those described for the native enzymes. Considering the increasing medical and pharmacological importance of oligosaccharides structures, this heterologous expression system will be useful to provide on a large scale the amount of glycosyltransferases required for chemoenzymatic synthesis. Toward this goal, the secretion of the three proteins in the culture medium represents a major advance with respect to production of enriched preparations. Moreover the recombinant fucT VI has been used for the synthesis of the sialyl Lewis x structure (31) and for the identification of a new inhibitor (Picasso *et al.*, manuscript in preparation). Recombinant ST6Gal has been compared with a recombinant ST6Gal expressed in CHO cells and shown to have similar kinetic properties (Ronin *et al.*, manuscript in preparation). The costs of *P. pastoris* fermentation being much lower than those for CHO cell culture, recombinant ST6Gal from *P. pastoris* may be an appropriate choice as a catalyst for remodeling therapeutic recombinant glycoproteins *in vitro*.

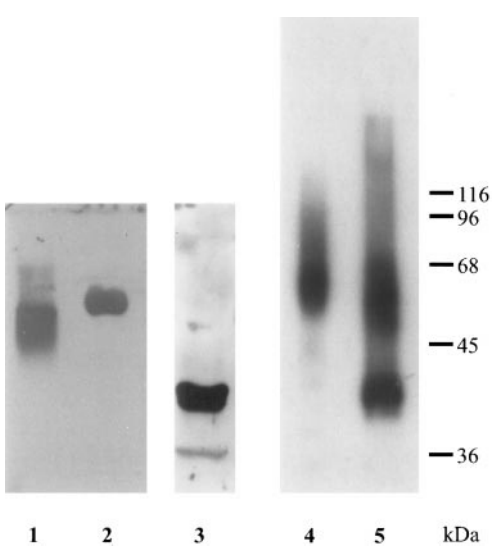

FIG. 2. Immunoblot analysis of rgal-T I, rST6Gal, and rfucT VI secreted in *P. pastoris* culture medium. 1, rgal-T I; 2, human gal-T I; 3, rST6Gal; 4, rfucT VI; 5, rfucT VI + pNGase F.

TABLE III

Michaelis Constants of Recombinant Gal-T I, ST6Gal, and FucT VI Expressed in *P. pastoris* KM71

	UDP-gal (μM)	GlcNAc (mM)
Human gal-T I	106[a]	3.3[a]
rgal-T I	143	3.8
	CMP-NeuAc (μM)	**LacNAc (mM)**
Rat ST6Gal	158[b]	1.67[b]
rST6Gal	150	2.3
	GDP-fuc (μM)	**LacNAc (mM)**
Human fucT VI	110[c]	4.9[c]
rfucT VI	170	7

[a] As determined in Malissard *et al.* (13).
[b] As determined in Krezdorn *et al.* (17).
[c] Legault *et al.* (27).

ACKNOWLEDGMENTS

This work was supported by Grant 5002-46084 of the Swiss National Science Foundation to EGB and Fluka Chemie AG. We thank Dr. Roland Wohlgemuth for continuous support.

REFERENCES

1. Varki, A. (1993) *Glycobiology* **3,** 97–130.
2. Yarema, K. J., and Bertozzi, C. R. (1998) *Curr. Opin. Chem. Biol.* **2,** 49–61.
3. Garegg, P. J. (1997) *Adv. Carbohydr. Chem. Biochem.* **52,** 179–205.

4. Watt, G. M., Lowden, P. A., and Flitsch, S. L. (1997) *Curr. Opin. Struct. Biol.* **7,** 52–60.
5. Herrmann, G. F., Wang, P., Shen, G. J., Garcia-Junceda, E., Khan, S. H., Matta, K. L., and Wong, C. H. (1994) *J. Org. Chem.* **59,** 6356–6362.
6. Fang, J., Li, J., Chen, X., Zhang, Y., Wang, J., Guo, Z., Zhang, W., Yu, L., Brew, K., and Wang, P. G. (1998) *J. Am. Chem. Soc.* **120,** 6635–6638.
7. Malissard, M., Zeng, S., and Berger, E. G. (1999) *Glycoconjugate J.* **16,** 125–139.
8. Joziasse, D. H., Shaper, N. L., Salyer, L. S., Van den Eijnden, D. H., Van der Spoel, A. C., and Shaper, J. H. (1990) *Eur. J. Biochem.* **191,** 75–83.
9. Gillespie, W., Kelm, S., and Paulson, J. C. (1992) *J. Biol. Chem.* **267,** 21004–21010.
10. Zeng, S., Dinter, A., Eisenkrätzer, D., Biselli, M., Wandrey, C., and Berger, E. G. (1997) *Biochem. Biophys. Res. Commun.* **237,** 653–658.
11. Herrmann, G. F., Krezdorn, C., Malissard, M., Kleene, R., Paschold, H., Weuster-Botz, D., Kragl, U., Berger, E. G., and Wandrey, C. (1995) *Protein Express. Purif.* **6,** 72–80.
12. Zigova, J., Mahle, M., Paschold, H., Malissard, M., Berger, E. G., and Weuster-Botz, D. (1999) *Enzyme Microb. Technol.* **25,** 201–207.
13. Malissard, M., Borsig, L., Di Marco, S., Grütter, M. G., Kragl, U., Wandrey, C., and Berger, E. G. (1996) *Eur. J. Biochem.* **239,** 340–348.
14. Romero, P. A., Lussier, M., Sdicu, A. M., Bussey, H., and Herscovics, A. (1997) *Biochem. J.* **321,** 289–295.
15. Hochstrasser, U., Lüscher, M., De Virgilio, C., Boller, T., and Wiemken, A. (1998) *FEBS Lett.* **440,** 356–360.
16. Gallet, P. F., Vaujour, H., Petit, J. M., Maftah, A., Oulmouden, A., Oriol, R., Le Narvor, C., Guilloton, M., and Julien, R. (1998) *Glycobiology* **8,** 919–925.
17. Krezdorn, C. H., Kleene, R. B., Watzele, M., Ivanov, S. X., Hokke, C. H., Kamerling, J. P., and Berger, E. G. (1994) *Eur. J. Biochem.* **220,** 809–817.
18. Borsig, L., Katopodis, A. G., Bowen, B. R., and Berger, E. G. (1998) *Glycobiology* **8,** 259–268.
19. Linder, S., Schliwa, M., and Kube-Granerath, S. (1996) *Biotechniques* **20,** 980–982.
20. Borsig, L., Ivanov, S. X., Herrmann, G. F., Kragl, U., Wandrey, C., and Berger, E. G. (1995) *Biochem. Biophys. Res. Commun.* **210,** 14–20.
21. Laemmli, U. K. (1970) *Nature* **227,** 680–685.
22. Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 4350–4354.
23. Watzele, G., Bachofner, R., and Berger, E. G. (1991) *Eur. J. Cell Biol.* **56,** 451–458.
24. Berger, E. G., Grimm, K., Bächi, T., Bosshart, H., Kleene, R., and Watzele, M. (1993) *J. Cell. Biochem.* **52,** 275–288.
25. Gill, G. S., Zaworski, P. G., Marotti, K. R., and Rehberg, E. F. (1990) *Biotechnology* **8,** 956–958.
26. Aikawa, J., Yamashita, T., Nishiyama, M., Horinouchi, S., and Beppu, T. (1990) *J. Biol. Chem.* **265,** 13955–13959.
27. Riederer, M. A., and Hinnen, A. (1991) *J. Bacteriol.* **173,** 3539–3546.
28. Legault, D. J., Kelly, R. J., Natsuka, Y., and Lowe, J. B. (1995) *J. Biol. Chem.* **270,** 20987–20996.
29. Grinna, L. S., and Tschopp, J. F. (1989) *Yeast* **5,** 107–115.
30. Schwientek, T., and Ernst, J. F. (1994) *Gene* **145,** 299–303.
31. Zeng, S., Gallego, R. G., Dinter, A., Malissard, M., Dudziak, G., Wandrey, C., Kamerling, J. P., and Berger, E. G. (1999) *Glycoconjugate J.*, in press.