BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **234,** 68–72 (1997)
ARTICLE NO. RC976580

The Golgi Sialoglycoprotein MG160, Expressed in Pichia pastoris, Does Not Require Complex Carbohydrates and Sialic Acid for Secretion and Basic Fibroblast Growth Factor Binding

YouJun Chen and Nicholas K. Gonatas
Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

Received March 31, 1997

MG160, a type I membrane sialoglycoprotein of the medial cisternae of the rat Golgi apparatus, shows high homology (over 90%) with CFR, a fibroblast growth factor receptor, and ESL-1, an E-selectin ligand of the cell surface of murine myeloid cells. When Chinese Hamster Ovary (CHO) cells were stably transfected with a cDNA lacking the transmembrane and C-terminus cytoplasmic domain of MG160 (ΔTMCT), a fully processed protein of 160 kDa apparent molecular mass was recovered in the culture medium. When these cells were treated with tunicymycin, a 130- to 140-kDa protein was immunoprecipitated from the culture medium. A construct lacking the signal sequence, the single transmembrane, and the cytoplasmic domains of MG160 (ΔTMCT-) was integrated at the HIS Pichia pastoris genome site using the expression vector pPIC 9 which possesses a yeast compatible signal sequence (Invitrogen). Recombinant protein accumulated in the medium to approximately 10 mg/L. The yeast recombinant protein lacked complex carbohydrates and sialic acid but bound ^{125}I bFGF. Similarly, rat MG160 subjected to deglycosylation by peptide:*N*-glycosidase F (PNGase) bound ^{125}I bFGF. © 1997 Academic Press

MG160 is a type I intrinsic membrane sialoglycoprotein of the medial cisternae of the rat Golgi apparatus (1,2). The protein contains complex carbohydrates and sialic acid as indicated by its resistance to Endoglucosaminidase H and F digestion, its sensitivity to neuraminidase digestion and its binding to the Limax flavus lectin (1).

The amino acid sequence of MG160, deduced from cDNA cloning and sequencing, revealed a polypeptide of 1,171 amino acids with an M_r of 133,403. The thirteen amino acid, positively charged C-terminal cytoplasmic tail of MG160 is followed by a single transmembrane domain and a large intralumenal domain containing 16 contiguous, approximately 60-residue-long, regularly spaced cysteine-rich repeats showing sequence identities ranging from 15 to 35%, and five potential NXT glycosylation sites. The lumenal domain of the protein contains a Pro-Gln rich segment followed by a cleavable signal peptide (3).

The gene of MG160 was assigned to human chromosome 16q22-23 by fluorescence *in situ* hybridization (4). The rat Golgi MG160 protein is virtally identical to CFR, a Fibroblast Growth Factor (FGF) binding protein of chicken, and to ESL-1, an E-selectin ligand of murine myeloid cell surfaces (5,6). It is noteworthy that MG160, isolated from rat brain, binds specifically ^{125}I bFGF (3). MG160 appears ubiquitously and exclusively in the Golgi apparatus of developing chicken embryos; the protein was first detected within the ectoblast and primitive endoblast prior to the formation of the primitive streak (7). The amino acid sequence of the human homologue of MG160 revealed an overall identity ranging from 88-95% with rat MG160, CFR, the Fibroblast Growth Factor binding protein in the chicken, and ESL-1, the ligand for E-selectin of mouse myeloid cell surfaces. The thirteen amino acids of the C-terminus cytoplasmic tail of MG160 are identical in the four species (4-6,8). The function of MG160 in the Golgi apparatus is not known. It was proposed that MG160 might be involved in the binding and regulation of secretion of those fibroblast growth factors (FGF) possessing signal sequences, such as the FGF3 (3,9).

The recognition that the amino acid sequence of MG160 is virtually identical to that of ESL-1, a plasma membrane protein of mouse myeloid cells, suggests that MG160 may be transported to the plasma membrane of certain cells in order to mediate interactions with other cells or molecules (6). In the case of ESL-1, these interactions are mediated by specific carbohy-

0006-291X/97 $25.00

drate recognition moieties including fucose residues which are necessary for E-selectin binding (6,10).

Many plasma membrane and internal membrane glycoproteins of mammalian cells contain a variety of sugar residues. However, the role of the carbohydrate moieties in MG160 and in other intracellular membrane glycoproteins is not completely understood (11). It was suggested that the carbohydrate moieties of plasma membrane glycoproteins are involved in "recognition events" which mediate cell adhesion, or binding to soluble ligands or microbes (11). It has also been proposed that carbohydrate moieties contribute to the proper folding and stability of glycoproteins (11,12).

Complex carbohydrates and sialic acic are obviously not necessary for the proper folding and exit of MG160 from the endoplasmic reticulum, since these modifications of the carbohydrate moieties occur in the medial to distal Golgi compartments (quoted in 13). However, complex carbohydrates and sialic acid may be needed for the stability and the potential translocation of MG160 from the Golgi apparatus to the plasma membrane and extracellular milieu. In the present experiments we used the Pichia pastoris expression system in order to produce recombinant protein and to examine the role of complex carbohydrates and sialic acid in FGF binding and secretion of MG160.

Since the introduction of Pichia pastoris as a suitable host system for the expression of heterologous proteins, several expression vectors and yeast strains were used for the production of a significant number of recombinant proteins (14,15). The two principal advantages of the Pichia pastoris system are high-levels of expression and ease of purification of the heterologous recombinant protein (14,15). However, the N-asparagine-linked olisaccharide chains secreted by Pichia pastoris are of the high mannose type, and the yeast lacks the enzymes required for the addition to glycoproteins of complex carbohydrates including sialic acid and fucose residues (15,16). Consequently, it is expected that MG160 expressed into the Pichia pastoris yeast should lack complex carbohydrates including sialic acid. Thus, the Pichia pastoris yeast becomes a suitable expression system to investigate the functional role of complex carbohydrates in MG160 and in other glycoproteins.

The experiments described in this paper are consistent with the conclusion that complex carbohydrates and sialic acid are not required for the stability, secretion and bFGF binding of the conserved medial Golgi sialoglycoprotein MG160.

MATERIALS AND METHODS

Bacterial and Yeast Strains

Pichia pastoris GS115 was used for the expression of MG160. E. Coli TOP10F′ was used for all plasmid constructions and propagation (Invitrogen).

Media Composition

Yeast Extract Peptone Dextrose Medium (YPD) consisted of 1% yeast extract, 2% peptone, and 2% dextrose. Minimal Glycerol Medium (MGY) consisted of 1.34% yeast nitrogen base (YNB), 1% glycerol, and 4×10^{-5} per cent biotin. Minimal Dextrose Medium (MD) consisted of 1.34% YNB, 4×10^{-5} per cent biotin, and 1% dextrose. Minimal Methanol Medium (MM) consisted of 1.34% YNB, 4×10^{-5} per cent biotin, and 0.5% Methanol.

Construction of Recombinant Pichia pastoris Carrying Rat MG160 cDNA Lacking the Signal Sequence, the Trans Membrane Domain, and the Cytoplasmic Tail of the protein (ΔTMCT−)

The full length cDNA of rat MG160 was subcloned from the Bluescript SK(−) vector into the Sca1-Not1 sites of the pBKCMV expression vector [pBKCMV-MG160] (Stratagene)(3). The construct lacking the cytoplasmic and membrane spanning domains and 3 contiguous amino acids of the lumenal domain of MG160 was designated as pBKCMV-ΔTMCT; this construct was created by established PCR techniques using pBKCMV-MG160 as template, 3-′primer 5′ 5′-TACAGTCTCAGCGGCCGCCTAGGATGTCATCACTTGC-3′, and 5′-primer 5′-CGCAACGACACTCTGCAGGA-3′. All constructs generated by PCR were verified by restriction enzyme digests. Subsequently, the signal sequence of MG160 was deleted by PCR using as the template the construct pBKCMV-ΔTMCT. The upstream forward primer used, 5′-ACGTAGAGTACTAAGATGGAATTCGTCCAGAATAGCCACGGTCAG-3′ contains the unique EcoR1 site. The internal reverse primer, 5′-GGTTCAGCTTGTAATTCCA-3′ used is located about 400bp downstream of the forward primer. A unique Xho1 site is located about 90bp upstream of the internal reverse primer. The PCR product was digested with both EcoR1 and Xho1 enzymes, and the resulting product was ligated into pBKCMV/ΔTMCT which had been digested with the same two enzymes. The insert(ΔTMCT−) of the resulting construct was released by EcoR1 and Not1 digestions. The ΔTMCT-construct was ligated into the EcoR1 and Not1 sites of the Pichia expression vector pPIC 9 which possesses a yeast compatible signal sequence located immediately upstream of the polylinker site (Invitrogen, San Diego, CA). The pPIC9/ΔTMCT-cDNA construct was integrated at the HIS 4 Pichia genome site after digestion with Sal1; this integration resulted in the generation of a His^+Mut^+ strain which can utilize methanol. The pPIC9/ΔTMCT-construct was digested with BstB1 for integration at the AOX1 site; this resulted in a His^+Mut^S strain which utilizes methanol slowly. The integrations of ΔTMCT-in both yeast strains were confirmed by PCR using the commercially available oligonucleotides, 5′-GACTGGTTCCAATTGACAAGC-3′ and 5′-GCAAATGGCATTCTGACATCC-3′ for the 5′ and 3′ end primers respectively (Invitrogen). Yeast clones were selected on the basis of the efficiency of expression of MG160 using SDS-PAGE of an aliquot of collected medium followed by Coomassie blue staining.

Expression of Recombinant Protein

A single colony of a His^+Mut^+ strain expressing ΔTMCT-was inoculated in 10 ml of MGY in a 100 ml baffled flask and grown at 30°C in a shaking incubator until the culture reached an OD_{600} of 2-6; the entire culture was then inoculated into 1 liter of MGY in a 4 liter baffled flask and grown at 30°C until the culture reached an OD600 of 2-6. The culture was then centrifuged at 2000×g for 5 min, and the cell pellet was resuspended in 100 ml of MM and incubated at 30°C for 20 hrs to induce the expression of the recombinant protein.

Quantitation of Secreted Protein

Quantitation of secreted recombinant MG160 was performed with SDS-PAGE, using bovine serum albumin as standard, and using the Bradford method for protein determination (17).

Glycosidase Digestions

Approximately 1-2 ug of recombinant MG160 and control rat MG160 were first denatured at 50°C for 3 min in 0.1 M sodium acetate pH 5.5, containing 0.1% SDS; subsequently a 6-fold excess of NP-40 was added to stabilize the endoglycosidase. The digestions were carried out at 37°C for 2 hrs with 5 mU each of the following enzymes: Neuraminidase (Genzyme, Boston, MA), Endoglucosaminidase H (ENDO H) and N-Glycosidase F(PNGase F)(Boehringer Mannheim, Germany), and terminated with the addition of Laemmli sample buffer and boiling (1,18,19). Deglycosylation was monitored by SDS-PAGE and subsequent transfer of the proteins to nitrocellulose paper and staining with a polyclonal antiserum against MG160 (20,21).

Inhibition of N-Asparagine Glycosylation by Tunicamycin

Chinese Hamster Ovary cells (CHO), were stably transfected with pBKCMV-ΔTMCT with the calcium phosphate method (22). The cells were treated overnight with 2ug/ml tunicamycin in complete medium at 37 °C. Tunicamycin is an irreversible inhibitor of the dolichol-phosphate (N-linked) glycosylation (23). Control sister cultures were treated identicaly in culture medium lacking tunicamycin. The next day the cells were radiolabeled for 3 hr at 37 °C with 150uCi/ml of [^{35}S] methionine/cysteine in a cysteine/methionine depleted medium. After labelling, the culture media were collected and cells were washed five times with PBS and then extracted in 500ul of lysis buffer (20mM Tris-HCl pH7.5, 150mM NaCl, 1%Triton X-100, 0.2% gelatin, 2mM EDTA, 1mM PMSF, 2ug/ml each of aprotinin, leupeptin and pepstatin). The cell extracts and culture media were centrifuged at 100,000g for 15 min to ensure removal of debris, and then precleared by rotation for 4 hr at 4 °C with 25 ul of packed agarose coupled to goat anti mouse IgG that had previously been incubated with an irrelevant monoclonal antibody The precleared extract was then incubated overnight at 4 °C with 300 ul of the rat monospecific mAb 10A8 before addition of 15ul of packed protein A-agarose beads. The Sepharose beads were then washed once in lysis buffer and five times in lysis buffer containing 0.5M NaCl. The immunocomplex was boiled in electrophoresis sample buffer and analyzed by SDS-PAGE followed by fluorography (19).

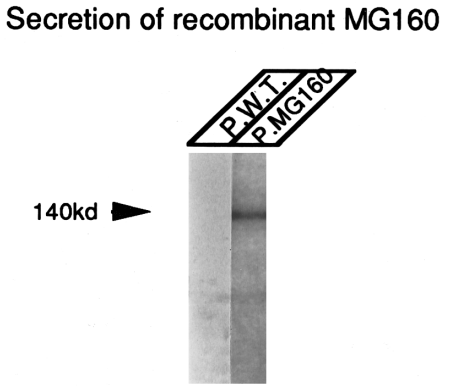

FIG. 1. Detection of secreted MG160 in culture media. One hundred ml of control culture media from the original yeast strain (P.W.T.) and media from yeast transfected with pPIC9/ΔTMCT-(P.MG160, a MG160 cDNA lacking the signal sequence, transmembrane, and cytoplasmic domains inserted in the pPIC9 vector) was concentrated to 1 ml. An aliquot of 1 ul, corresponding to 1 microgram of recombinant protein, was subjected to SDS-PAGE and stained with Coomassie blue. The recombinant protein displayed an apparent molecular mass of 140 kDa.

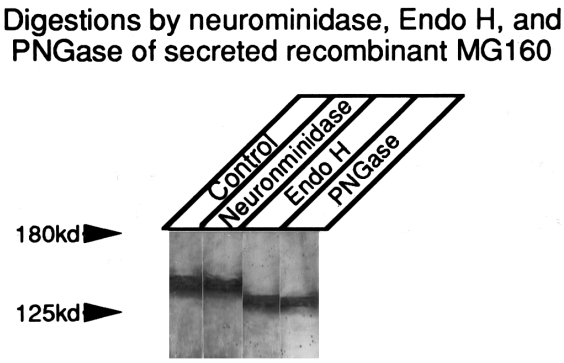

FIG. 2. Enzyme digestions. One to two micrograms of the recombinant protein produced by Pichia was digested with Endo H, PNGase, and neuraminidase, transferred to nitrocellulose, and immunoblotted with an antiserum against MG160 (21). While the neuraminidase digestion did not alter the mobility of the recombinant protein, both Endo H and PNGase digestions resulted in faster moving bands at approximately 130 kDa. In the control lane the recombinant protein was treated identically except for the absence of enzymes in the incubation medium.

^{125}I bFGF Binding

One to two ug of rat brain MG160 protein obtained by immunoaffinity chromatography with mAb 10A8 (1) and the same amount of recombinant MG160 protein from the yeast culture media were first subjected to SDS-PAGE electrophoresis and then transferred to nitrocellulose according to Laemmli, and Towbin et al. (19,20). The nitrocellulose paper was blocked for 1 hr in TBST (10mM Tris, pH7.4, 0.15 M NaCl, and 0.1% Tween) containing 3% fish gelatin(FG); each paper was then incubated for 90 min at room temperature with 100pM of ^{125}I bFGF, a gift from Dr. Thomas Maciag, Holland Laboratory American Red Cross, Rockville, MD; the nitrocellulose paper was then washed 4 times with 3% FG in TBST. The paper was exposed on film for an average of 7-14 days at −80 °C. To confirm the specificity of bFGF binding, 1-2 ug of the recombinant MG160 was blocked with 1000-fold molar excess of non-radioactive bFGF and washed extensively prior to incubation with ^{125}I bFGF(3).

RESULTS

Collection of culture media and recovery of recombinant MG160 secreted by Pichia (P.MG160). After incubation of transformed yeast cells in 100 ml of minimal methanol medium at 30 °C for 20 hr, the culture medium was collected and concentrated in a Centricon 100 (Amicon) to 1 ml. Approximately one milligram of recombinant protein was recovered from 100 ml of culture media. SDS-PAGE electrophoresis of the recovered protein followed by Coomassie blue staining revealed a single band of approximately 140 kDa (Fig.1). After Endo H and PNGase digestions, the recombinant protein migrated as a single 130 kDa band, while the migration of the protein was not affected by neuraminidase digestion (Fig.2). These results indicate that the recombinant MG160 does not contain complex carbohydrates and sialic acid. It is noteworthy that both the recombinant MG160 protein and rat MG160 subjected

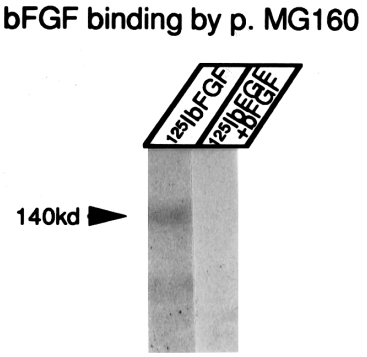

FIG. 3. The Pichia recombinant protein lacking sialic acid and complex carbohydrates binds bFGF. One ug of protein secreted by Pichia was subjected to SDS-PAGE, transfered to nitrocellulose, and incubated for 90 min at room temperature with 100pM ^{125}I bFGF, washed, and processed for fluorography; a single 140 kDa radioactive band was detected. The radioactive band was not present when the recombinant protein was incubated with a thousand-fold excess of unlabeled bFGF prior to incubation with ^{125}I bFGF.

to PNGase digestion specifically bound ^{125}I bFGF (Figs.3, 4). These results are consistent with the conclusion that complex carbohydrates and sialic acid are not required for the binding of MG160 to bFGF.

In order to further examine the role of N-linked sugars in the secretion of MG160 lacking the transmembrane and cytoplasmic domains (pBKCMV/ΔTMCT), CHO cell stable transfectants were treated with tunicamycin (23). Immunoprecipitations of control cell lysates with mAb 10A8, labeled for 3 hr with ^{35}S-trans, produced a 160 kDa band, corresponding to mature, fully glycosylated, MG160, and a 140 kDa band, corresponding to immature, or incompletely processed, MG160 (Fig.5, -Tun. Cells). In the culture media of these cells, only the mature 160 kDa form of the protein was immunoprecipitated (Fig. 5,-Tun. Media). In contrast, in the tunicamycin treated cells, a single, approximately 140 kDa band was detected in both cell lysates and media (Fig. 5, + Tun. Cells, Media).

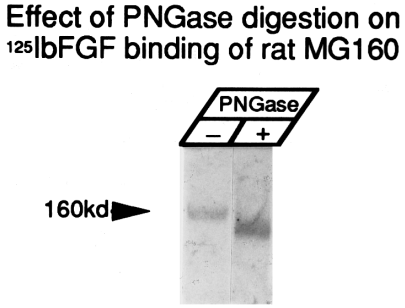

FIG. 4. Digestion of rat MG160 with PNGase does not inhibit the binding with ^{125}I bFGF. One ug of affinity purified rat MG160 was subjected to SDS-PAGE, transferred to nitrocellulose, and incubated with ^{125}I bFGF as in Fig.3. Both enzyme digested and undigested MG160 binds to bFGF.

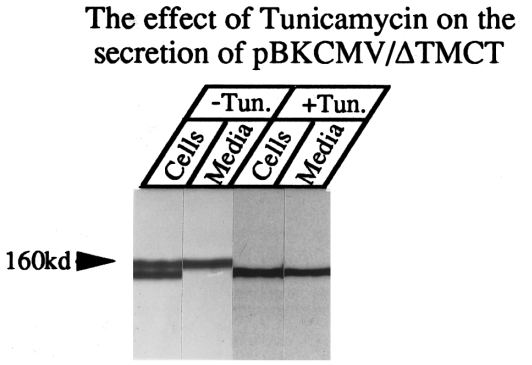

FIG. 5. Effect of tunicamycin on the secretion of pBKCMV/ΔTMCT by CHO cells. Chinese hamster ovary cells (CHO) were stably transfected with pBK-CMV-ΔTMCT, a construct lacking the transmembrane and cytoplasmic domains of the cDNA of MG160. Cells were metabolically labeled for 3 hrs at 37°C and cell lysates (Cells) and culture media (Media) were immunoprecipitated with mAb 10A8 and subjected to SDS-PAGE and fluorography. In the absence of tunicamycin (-Tun.) a 160 kDa band representing mature MG160 was found in the media, while cell lysates displayed both a mature 160 kDa protein and an immature precursor of about 130-140 kDa apparent molecular mass. In cells treated with tunicamycin overnight, the secreted and intracellular protein displayed a mobility of 130-140 kDa, identical to that of the immature precursor found in cells not treated with tunicamycin.

DISCUSSION

The amount of MG160 secreted by Picia pastoris, approximately 10 mg/liter, is considerably lower than that of other recombinant proteins such as the tick anticoagulant peptide (1.7g/l) or the mouse epidermal growth factor (EGF) (0.45g/l) (24,25). The low expression level of MG160 by the Pichia pastoris yeast is probably due to the size of the recombinant protein which consists of 1134 amino acids, in contrast to the significantly shorter tick anticoagulant peptide and the mouse EGF (24,25). We cannot exclude the possibility that intracellular or extracellular proteolytic cleavages have contributed to the low yields of MG160; however, western blotting of culture media did not reveal breakdown products, and the MG160 was quite stable after neuraminidase digestion (Fig.2). The preparation and selection of Pichia pastoris transformants with many copies of the MG160 gene may increase the yield of the recombinant protein.

Yeast glycoproteins are of the high mannose type and heterologous proteins expressed in yeast are hyperglycosylated with an average mannose chain length of 50-100 residues in Saccharomyces cerevisiae, and of 8-14 residues in Pichia pastoris (16). Therefore, it is not surprising that the recombinant MG160 lacked complex carbohydrates and sialic acid (Fig. 2). However, it is noteworthy that the lack of N-linked glycosylation of the recombinant MG16O and of the MG160 protein expressed in stable CHO transfectants treated with tunicamycin did not affect their secretion (Figs. 2,5).

An intriguing property of MG160 is its ability to bind bFGF in vitro (3,5). The functional implications of this observation are not known. However, cells may need an FGF binding protein in the Golgi apparatus, in another intracellular compartment or at the cell surface, in order to regulate FGF secretion and/or binding to the FGF receptor involved in signal transduction. Lately, Zhou and colleagues reported that CFR, the chicken homologue of the rat MG160, binds FGFs in the absence of heparan sulfate which is indispensable for the binding of FGF with its functional receptor, FGFR (26). Furthermore, they have identified a CFR fragment of 450 residues ($CFR_{290-740}$) that is responsible for the heparan independent binding of CFR to bFGF. This segment of the CFR contains two NXT potential glycosylation sites (at $CFR_{447-456}$ and $CFR_{582-591}$) (5,26). The results of the present study suggest that the N-glycosylation of MG160 is not required for bFGF binding. The feasibility and availability of the Pichia recombinant MG160 protein may facilitate structural studies on the protein including an assessment of the role of N-linked sugar moieties in FGF binding.

ACKNOWLEDGMENT

Supported by a Javits Neuroscience Award (NS05572) from the National Institute of Neurological Disorders and Stroke of the USPHS.

REFERENCES

1. Gonatas, J. O., Mezitis, S. G. E., Stieber, A., Fleischer, B., and Gonatas, N. K. (1989) *J. Biol. Chem.* **264,** 646–653.
2. Singer, S. J. (1990) *Annu. Rev. Cell Biol.* **6,** 247–296.
3. Gonatas, J. O., Mourelatos, Z., Stieber, A., Lane, S. W., Brosius, J., and Gonatas, N. K. (1995) *J. Cell Sci.* **108,** 457–467.
4. Mourelatos, Z., Gonatas, J. O., Nycum, L. M., Gonatas, N. K., and Biegel, J. (1995) *Genomics* **28,** 354–355.
5. Burrus, L. W., Zuber, M. E., Lueddecke, B. A., and Olwyn, B. B. (1992) *Mol. Cell Biol.* **12,** 5600–5609.
6. Steegmaier, M., Levinowitz, A., Isenmann, S., Borges, E., Lenter, M., Kocher, H. P., and Vestweber, D. (1995). *Nature* **373,** 55–63.
7. Stieber, A., Mourelatos, Z., Chen, Y.-J., Le Douarin, N., and Gonatas, N. K. (1995) *Exp. Cell Res.* **219,** 562–570.
8. Mourelatos, Z., Gonatas, J. O., Cinato, E., and Gonatas, N. K. (1996) *DNA Cell Biol.* **15,** 1121–1128.
9. Kiefer, P., Peters, G., and Dickson, C. (1991) *Mol. Cell Biol.* **13,** 5781–5793.
10. Crocker, P. R., and Feizi, T. (1996). *Curr. Opin. Struct. Biol.* **6,** 679–691.
11. Gahmberg, C. G., and Tolvanen, M. (1996) *TIBS,* 308–311.
12. Wang, C., Eufemi, M., Turano, C., and Giartoso, A. (1996) *Biochemistry* **35,** 7699–7307.
13. Johnston, P. A., Stieber, A., and Gonatas, N. K. (1994) *J. Cell Sci.* **107,** 529–537.
14. Cregg, J. M., Barringer, K. J., Hessler, A. Y., and Madden, K. R. (1985) *Mol. Cell Biol.* **12,** 3376–3385.
15. Cregg, J. M., Vedvick, T. S., and Raschke, W. (1993) *Biotechnology* **11,** 905–909.
16. Eckart, M. R., and Bussineau, C. M. (1996) *Curr. Opin. Biotechnol.* **7,** 525–530.
17. Bradford, M. (1976). *Annu. Biochem.* **72,** 248–254.
18. Tarentino, A. L., Gomez, C. M., and Plummer, T. H. (1985) *Biochemistry* **24,** 4665–4671.
19. Laemmli, U. K. (1970) *Nature* **227,** 680–685.
20. Towbin, H. T., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 4350–4354.
21. Croul, S., Mezitis, S. G. E., Stieber, A., Chen, Y.-J., Gonatas, J. O., Good, B., and Gonatas, N. K. (1990) *J. Histochem. Cytochem.* **38,** 957–963.
22. Wigler, M., Silverstein, S., Lee, L-S., Pellicer, A., Cheng, Y-C., and Axel, R. (1977) *Cell* **11,** 223–232.
23. Duksin, T., and Mahoney, W. C. (1981) *J. Biol. Chem.* **257,** 3105–3109.
24. Laroche, Y., Storme, V., De Meutter, J., Messens, J., and Lauwereys, M. (1994) *Biotechnology* **12,** 1119–1124.
25. Clare, J. J., Romanos, M. A., Rayment, F. B., Rowedder, J. E., Smith, M. A., Payne, M. M., Sreekrishna, K., and Henwood, C. A. (1991) *Gene* **105,** 205–212.
26. Zhou, Z., Zuber, M. E., Burrus, L. W., and Olwin, B. B. (1997) *J. Biol. Chem.* **272,** 5167–5174.