Tetracycline-Regulated Overexpression of Glycosyltransferases in Chinese Hamster Ovary Cells

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Received 3 December 1998; accepted 13 June 1999

Abstract: The glycosylation patterns of recombinant therapeutic glycoproteins can be engineered by overexpression of glycosyltransferases in the host cells used for glycoprotein production. Most prior glycosylation engineering experiments have involved constitutive expression of cloned glycosyltransferases. Here we use tetracycline-regulated expression of two glycosyltransferases, *N*-acetylglucosaminlytransferases III and V (GnTIII and GnTV) to manipulate glycoform biosynthesis in Chinese hamster ovary (CHO) cells and to study the effect of glycosyltransferase overexpression on this host. The amount of GnTIII and GnTV in these cells, and the glycosylation patterns of several cellular glycoproteins, could be controlled simply by manipulating the concentration of tetracycline in the culture medium. Using this system, it was found that overexpression of either GnTIII or GnTV to high levels led to growth inhibition and was toxic to the cells, indicating that this may be a general feature of glycosyltransferase overexpression. This phenomenon has not been reported previously, probably due to the widespread use of constitutive promoters, and should be taken into account when designing vectors for glycosylation engineering. The growth inhibition effect sets an upper limit to the level of glycosyltransferase overexpression, and may thereby also limit the maximum extent of in vivo modification of poorly accessible glycosylation sites. Also, such inhibition implies a bound on constitutive glycosyltransferase expression which can be cloned. *Biotechnol Bioeng* **65**: 542–549, 1999.

Keywords: Chinese hamster ovary cells; glycosylation engineering; tetracycline-regulated expression; glycosyltransferase; growth-inhibition

INTRODUCTION

Improved versions of therapeutic glycoproteins may be produced by manipulation of their glycosylation patterns (Bailey, 1991; Bailey et al., 1998; Stanley, 1992). For example, "second generation" EPO and tPA glycoproteins, obtained through changes in the glycosylation pattern of earlier versions, are now advancing through clinical trials. The new versions of these two drugs were produced, respectively, by enrichment of superior glycoforms during the purification of the final product (Fürst, 1997), and by introduction of mutations in the polypeptide chain, which shifted the position of the oligosaccharide within the protein and led to a different glycosylation pattern (Keyt et al., 1994). In both cases, biosynthesis of the superior glycoforms was achieved with the standard glycosylation apparatus of normal Chinese hamster ovary (CHO) cells.

An alternative, complementary route for production of improved glycoproteins is through genetic manipulation of the host glycosylation pathway (Bailey, 1991). New glycoforms or glycoform distributions can be generated by introduction of glycosyltransferase and glycosidase genes into the host cells, or by antisense inhibition of an endogenous glycosyltransferase activity (Bailey et al., 1998; Prati et al., 1998). This is an attractive route for synthesis of new glycoforms since it does not add major costs to the process, and the required stable genetic changes can be introduced into the host cells using reliable and efficient technology already tested for production of glycoprotein drugs.

Previous work in this field (reviewed in Bailey et al., 1998) has only used constitutive expression of the glycosyltransferase genes, and little attention has been paid to the expression level of these enzymes. However, this variable could play an important role when trying to maximize the proportions of beneficial glycoforms within the glycoform population of a recombinant protein (Umaña and Bailey, 1997). In this work we used tetracycline-regulated expression (Gossen and Bujard, 1992) of glycosyltransferase genes as a means to control glycoform biosynthesis in CHO cells and studied the effect of glycosyltransferase overexpression on the host cells.

Two different glycosyltransferases were used, *N*-acetylglucosaminyltransferase III (GnTIII) and *N*-acetylglucosaminyltransferase V (GnTIII). Both of these Golgi-localized enzymes participate in the N-linked biosynthetic pathway (reviewed in Schachter, 1986). GnTIII catalyzes the transfer of an *N*-acetylglucosamine (GlcNAc) residue to the bisecting position of N-linked oligosaccharides, leading to bisected hybrid and bisected complex oligosaccharides (Fig. 1a). The latter type of carbohydrates have been implicated in the biological activity of therapeutic antibodies (Lifely et al., 1995) and regulating the expression level of GnTIII in CHO cells could be important when trying to maximize the

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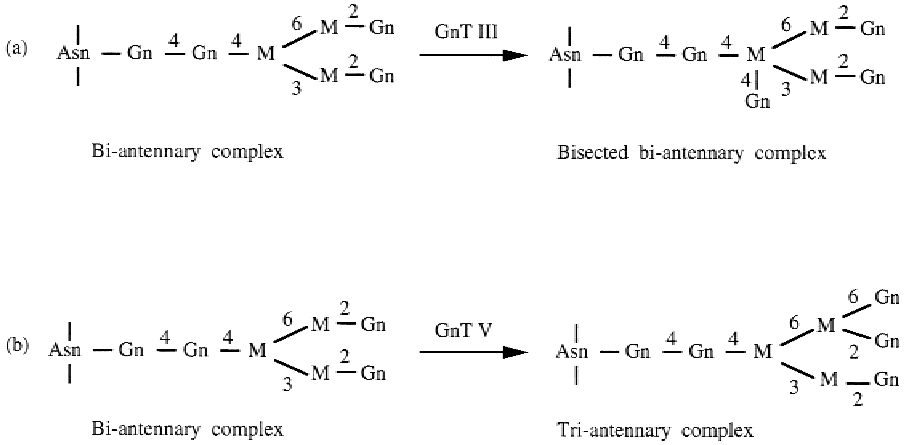

Figure 1. Reactions catalyzed by GnTIII and GnTV on common N-linked, bi-antennary complex oligosaccharide substrates. M stands for mannose and Gn, *N*-acetylglucosamine (GlcNAc).

synthesis of therapeutic antibody glycoforms carrying bisected complex oligosaccharides (Umaña and Bailey, 1997). GnTIII is normally not expressed in wild-type CHO cells (Stanley and Campbell, 1984). GnTV catalyzes the transfer of a GlcNAc to the α6-mannose arm of biantennary or tri-antennary complex oligosaccharides leading to tri- and tetra-antennary complex compounds (Fig. 1b). Higher oligosaccharide antennarity of EPO leads to an increase of its in vivo activity, apparently due to reduced kidney filtration (Misaizu et al., 1995), and may be investigated as a means to increase the circulatory half-life of other small glycoprotein drugs.

MATERIALS AND METHODS

Mammalian Cell Lines and Media

CHO-DUKX cells were cultured in FMX-8 medium (Cell Culture Technologies, Switzerland) supplemented with 10% (v/v) fetal calf serum (Boehringer Mannheim), 3 g/L HEPES and 1% (v/v) antibiotic/antimycotic solution (Gibco). Cells were grown as monolayers in stationary T-flasks, using 0.2 mL of medium/cm^2 of culture surface. The cultures were maintained in an incubator at 37°C under a 5% CO_2 atmosphere. For subculturing, cells were detached from T-flasks by addition of Cell Dissociation Solution (Sigma).

Plasmids

Plasmid vectors pUHD15-1, for constitutive expression of the tetracycline-transactivator (tTA); pUHD10-3, for tetracyline-regulated expression of any inserted gene; and pUGH16-3, for tetracycline regulated expression of β-galactosidase (lacZ) (Gossen and Bujard, 1992) were obtained from Dr. Hermann Bujard (University of Heidelberg). Plasmid vectors pBluescriptIIKS(+)-GnTIII, carrying the rat GnTIII cDNA (Nishikawa et al., 1992), and pSKV3-GnTV, carrying the human GnTV cDNA (Saito et al., 1995), were obtained from Dr. Naoyuki Taniguchi (University of Osaka, Japan). Plasmid vectors pSV2Neo and pPur, for constitutive expression of genes conferring resistance to neomycin and puromycin respectively, were purshased from Clontech.

Construction of Glycosyltransferase Expression Vectors

C-myc epitope-encoding DNA (Nilsson et al., 1993) was added to the 3,′ end of the GnTIII cDNA by PCR amplification. The product was subcloned into pBluescriptIIKS(+) (Stratagene) for sequencing, and finally subcloned into pUHD10-3 to generate plasmid vector pUHD10-3-GnTIIIm. The GnTV cDNA was directly subcloned into pUHD10-3 to generate plasmid vector pUHD10-3-GnTV.

The c-myc epitope-encoding sequence was added by a two-step sequential PCR approach. The same forward primer was used in both PCR steps. It hybridizes with the unique *Nde*I restriction site towards the downstream end of the GnTIII cDNA, and included a *BamH*I site for subcloning. Its sequence was 5′- ctcgaaggatcccttcgccttccatatgc. The first reverse primer hybridized with the GnTIII carboxy-terminus-encoding sequence and included the first 22 bases coding for the c-myc epitope tag. Its sequence was 5′-cagagatcagcttttgttccgggccctccgttgtatccaactt-3′. The second reverse primer contained the entire human c-myc epitope-(EQKLISEEDL) encoding sequence separated from the GnTIII carboxy-terminus-encoding sequence by an additional proline codon, and included the stop codon plus an *Xba*I site for subcloning. Its sequence was 5′-gtgtgttctagactacaggtcttcttcagagatcagcttttgttccgg-3′.

The final PCR product was subcloned into pBluescriptIIKS+ and sequenced by cycle sequencing to check if any mutations had been introduced. The remaining upstream portion of the GnTIII cDNA was subcloned into this plasmid, then the reassembled, modified cDNA was subcloned into plasmid vector pUHD10-3 between the *Eco*RI and *Xba*I

sites. To construct the other glycosyltransferase expression vector, GnTV cDNA was recovered from vector pSKV3-GnTV using *Xba*I and partial *Eco*RI digestions and subcloned directly into pUHD10-3.

SDS–PAGE and Electroblotting

Cells dissociated from culture plates were harvested by centrifugation at 400*g* for 5 min and washed with 0.5 mL cold PBS. The cell pellet was resuspended in 30 μL of lysis buffer containing 50 m*M* MOPS–NaOH, 2% (v/v) Triton X-100, 1 m*M* $MgCl_2$, 1 m*M* dithiotreitol, 10% (w/v) sucrose, and 150 m*M* NaCl and subjected to sonification. Insoluble material was removed by centrifugation at 1000*g* for 10 min at 4°C. Equal amounts of protein lysate were prepared using the standard BCA protein assay (pierce). Generally 5 μg of lysate were prepared for SDS–PAGE and subjected to electrophoresis through a 8.75% polyacrylamide gel under reducing conditions. The resolved proteins in the gel were electroblotted to a PVDF membrane (ImmobilonPSQ, Millipore) and the membranes were then probed with either antibody or lectins as described below.

Western and Lectin Blotting

Nonspecific binding sites on the PVDF membrane were blocked by overnight incubation at 4°C with 0.5% (w/v) blocking reagent (Boehringer Mannheim) in Tris-buffered saline (TBS). Membranes were washed 2 or 3 times for 10 min with TBS containing 0.1% (v/v) Tween 20 (TBS-T).

For analysis with lectins, the TBS containing 1 m*M* $MgCl_2$, 1 m*M* $MnCl_2$, and 1 m*M* $CaCl_2$ (lectin buffer) was used for the third wash. Membranes were then incubated for 1 h at room temperature with a solution of biotinylated E-PHA (Oxford Glycosciences) at a concentration of 25 μg/mL in lectin buffer or L-PHA-digoxigenin (Boehringer Mannheim) at 50 μg/mL in the same buffer.

For western analysis, membranes were incubated for 1 h at room temperature with the anti-c-myc monoclonal antibody 9E10 (a gift from Prof. Dr. J. A. Robinson, University of Zürich) at a concentration of 2 μg/mL in TBS-T (GnTIII western blots) or with an anti-GnTV rabbit polyclonal antibody (a gift from Dr. M. Pierce, University of Georgia, USA) diluted 1000-fold in TBS-T (GnTV western blots).

Membranes were washed three times with TBS-T and incubated for 1 h with either anti-biotin-alkaline phosphatase (Boehringer Mannheim) diluted 1000-fold in TBS-T (detection via E-PHA), anti-digoxigenin-alkaline phosphatase (Boehringer Mannheim) diluted 1000-fold in TBS-T (detection via L-PHA), anti-mouse IgG-horse radish peroxidase (Amersham) diluted 10,000-fold in TBS-T (detection of GnTIII), or anti-rabbit IgG-horse radish peroxidase (Amersham) diluted 10,000-fold in TBS-T (detection of GnTV). Membranes were subsequently washed three times with TBS-T. For analysis with lectins, the membrane was washed one more time with 0.1 *M* Tris, 0.05 *M* $MgCl_2$, and 0.1 *M* NaCl, pH 9.5 then incubated with the same buffer plus 0.375% (v/v) X-phosphate (Boehringer Mannheim) and 0.5% (v/v) NBT (Boehringer Mannheim). When color developed, the membranes were washed with water and air dried. For western analysis, bound antibody was detected using an enhanced chemiluminescence kit (ECL kit, Amersham) following the manufacturer's instructions.

Transfection of DNA into CHO Cells

DNA, either complexed with cationic liposomes (LipofectamineTM, Gibco), or co-precipitated with hydroxyapatite (calcium phosphate DNA transfection method), was transfected into CHO cells. Transfections using LipofectamineTM were carried out according to the manufacturer's instructions. Calcium phosphate DNA transfections were carried out using an efficient version of this method (Jordan and Wurm, 1996) with some additional modifications. Briefly, 24 h before transfection cells were seeded to approximately 40% confluency in a T25 flask and incubated at 37°C overnight. The next day the medium was replaced with fresh culture medium 1 h before transfection. For each flask with cells to be transfected, a solution of DNA, $CaCl_2$, and water was prepared by mixing 15 μg of total plasmid vector DNA, 75 μL of a 1 *M* solution of $CaCl_2$, and adding water to a final volume of 150 μL. To this solution, 150 μL of a 50 m*M* HEPES, 280 m*M* NaCl, 1.5 m*M* Na_2HPO_4 solution at pH 7.05 was added, mixed by vortexing for 5 s, and left to stand at room temperature for 25 s. This brief incubation led to the formation of very fine DNA/hydroxyapatite co-precipitates, which were then diluted with 5 mL of culture medium containing 2% (v/v) fetal calf serum (FCS). The resulting suspension was added to the cells in the T25 flask in place of the existing culture medium, and the cells were incubated for 5 h at 37°C. The medium was then replaced with 5 mL of a 15% (v/v) glycerol solution in culture medium containing 2% (v/v) FCS and the cells were left for 30 s at room temperature, before a final medium exchange to 5 mL of culture medium containing 10% (v/v) FCS. The cells were then incubated at 37°C until assayed or transferred to other culture flasks or dishes.

Generation of CHO Cells Expressing the Tetracycline-Transactivator (tTA)

CHO cells were co-transfected with pUHD15-1 and pSV2Neo by lipofection. Two days after transfection, the cells were transferred to a T75 flask, and Gentamycin (G418, Boehringer Mannheim) was added to the culture medium at a concentration of 400 mg/mL. The medium was replaced every 3 days for 2 weeks until G418 resistant clones had grown. The cells were resuspended and individual cells were transferred to wells of a 96-well cell culture plate using a cell sorter (FACS Star Plus, Beckton Dickenson). The clones were then screened for adequate levels of tTA expression. Tetracycline-regulated expression of a β-galactosidase gene was used as a marker for tTA

expression as described below. Clones were seeded in duplicate in 6-well plates and transiently transfected by lipofection with vector pUHG16-3. After transfection, 1 μg/mL tetracycline (final concentration) was added to one of each pair of transfectants, and the cells were incubated for 72 h at 37°C. The intracellular level of β-galactosidase activity in each transfectant was then measured using ONPG (Sigma) as a substrate (Sambrook et al., 1989). The clone with the highest level of β-galactosidase activity in the absence of tetracycline, named CHO-tTA, was chosen for further work.

Generation of CHO Cells with Stable, Tetracycline-Regulated Expression of GnTIII and GnTV

Two T-25 flasks were seeded with CHO-tTA cells to approximately 40% confluency and incubated at 37°C overnight. The next day, the cells in one flask were co-transfected with vectors pUHD10-3-GnTIIIm and pPur, and in the other with vectors pUHD10-3-GnTV and pPur, using the calcium phosphate transfection method. The DNA used for transfections was linearized, pUHD10-3-based expression vectors with *Pvu*I and pPur with *Pvu*II. The linearized vectors were co-transfected in a molar ratio of 15:1 of pUDH10-3-GnTIIIm:pPur or pUDH10-3-GnTV:pPur, and the total amount of DNA used per transfection was 15 μg. Immediately after transfection, tetracycline was added to the culture medium to a concentration of 2 μg/mL. Two days after transfection puromycin (Clontech) was added to the culture medium to a concentration of 7.5 μg/mL. The medium was replaced every 3 days until puromycin resistant clones of adequate size had grown. Thirty individual clones for each glycosyltransferase were transferred to a 96-well culture plate. The clones were grown in the presence of tetracycline and then screened for glycosyltransferase expression as described below.

For screening, clones were grown in duplicate in 6-well plates. One set of wells were cultured in the absence of tetracycline for 4 days, while the other cells were cultured in the presence of 2 μg/mL of tetracycline. After this incubation period, the approximate level of confluency was estimated and the cells harvested, lysed, and analyzed for tetracycline-regulated expression of the respective glycosyltransferase using the SDS–PAGE and western blot procedures described above. Lectin binding to cellular glycoproteins was used to visualize the extent of modification by the heterologous glycosyltransferase. Clones with high levels of expression in the absence of tetracycline and low levels of expression in the presence of tetracycline were selected, expanded, and stocks were frozen. Clonal purity was ensured by subcloning from a single clone for each glycosyltransferase by limited dilution, giving clones CHO-tet-GnTIIIm and CHO-tet-GnTV respectively, which were used for further work.

RESULTS AND DISCUSSION

Establishment of CHO Cell Lines with Tetracycline-Regulated Overexpression of Glycosyltransferases

The strategy used for establishment of glycosyltransferase overexpressing cell lines consisted of first generating an intermediate CHO cell line constitutively expressing the tetracycline-controlled transactivator (tTA) at an adequate level for the tetracycline-regulated expression system to work well (Yin et al., 1996). The tTA level had to be sufficiently high to activate high levels of transcription, in the absence of tetracycline, from the minimal promoter upstream of the glycosyltransferase genes. CHO cells were co-transfected with a vector for constitutive expression for tTA, driven by the human cytomegalovirus (hCMV) promoter/enhancer, and a vector for expression of a neomycin-resistance (NeoR) gene. An excess of the tTA-expression vector was used and neomycin-resistant clones were isolated.

The clones were screened using a functional assay for tTA expression (Gossen and Bujard, 1992; Yin et al., 1996). This was done by transfection of each clone with a second vector harboring a reporter gene, lacZ, under the control of the tet-promoter and screening for tetracycline-regulated (tet-regulated), transient expression (i.e., 1–3 days after transfection) of β-galactosidase activity. CHO-tTA, which showed the highest level of tet-regulated β-galactosidase activity among 20 screened clones, was selected for further work.

CHO-tTA cells were tested for tet-regulated expression of GnTIII by transfecting the cells with vector pUHD10-3-GnTIIIm and comparing the relative levels of GnTIII after incubation of the cells in the presence and absence of tetracycline for 48 h. GnTIII levels were compared by western blot analysis, using a monoclonal antibody (9E10) which recognizes the c-myc peptide epitope tag at the carboxy-terminus of GnTIII. The tag had been introduced through a modification of the glycosyltransferase gene using PCR amplification. Various reports have demonstrated addition of peptide epitope tags to the carboxy-termini of glycosyltransferases, a group of enzymes sharing the same topology, without disruption of localization or activity (Nilsson et al., 1993; Rabouille et al., 1995). Figure 2 shows that in clone CHO-tTA GnTIII accumulation is significantly higher in the absence than in the presence of tetracycline. The range of tetracycline concentrations over which GnTIII expression can be quantitatively controlled was found to be from 0 to 100 ng/mL. This result agrees with previous research using different cell lines and genes (Yin et al., 1996).

To generate a stable cell line with tet-regulated expression of GnTIII, CHO-tTA cells were co-transfected with vector pUHD10-3-GnTIIIm and vector, pPUR, for expression of a puromycin resistance gene. In parallel, CHO-tTA cells were co-transfected with pUHD10-3-GnTV and pPUR vectors to generate an analogous cell line for this other

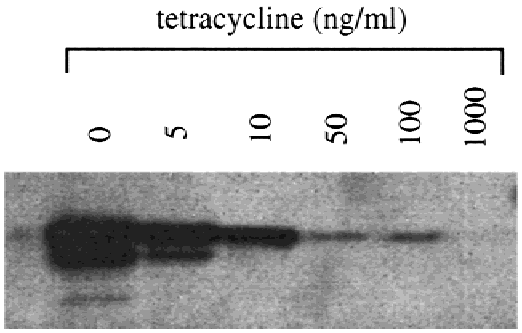

Figure 2. Western blot analysis of tetracycline-regulated expression of GnTIII. CHO-tTA cells were transfected with the pUDH10-3-GnTIIIm expression vector and then cultured for 48 h in the presence of the indicated concentrations of tetracycline. Cell lysates were then preprared for western blot analysis probing with an antibody (9E10), which recognizes specifically the c-myc tag added to GnTIII at its carboxy-terminus.

glycosyltransferase. A highly efficient calcium phosphate transfection method was used and the DNA was linearized at unique restriction sites outside the eucaryotic expression cassettes. By using a host in which the levels of tTA expressed had first been proven to be adequate, the probability of finding clones with high expression of the glycosyltransferases in the absence of tetracycline is increased.

Stable integrants were selected by puromycin resistance, keeping tetracycline in the medium throughout clone selection to mantain glycosyltransferase expression at basal levels. For each glycosyltransferase, 16 puromycin resistant clones were grown in the presence and absence of tetracycline, and 8 of each were analyzed by western blot analysis (Fig. 3). The majority of the clones showed good regulation of glycosyltransferase expression. One of the GnTIII-expressing clones showed a relatively high basal level in the presence of tetracycline (Fig. 3B, clone 3), which suggests integration of the expression cassete close to an endogenous CHO-cell enhancer; while two puromycin-resistant clones showed no expression of GnTIII in the absence of tetracycline (Fig. 3B, clones 6 and 8). Among the clones showing good regulation of expression, different maximal levels of glycosyltransferase were observed. This may be due to variations in the site of integration or number of copies integrated.

In vivo activity of the glycosyltransferases was verified by E-PHA and L-PHA lectin binding to endogenous cellular glycoproteins derived from various clones grown in the presence and absence of tetracycline (Fig. 4). E-PHA lectin binds to bisected oligosaccharides, the products of GnTIII-catalyzed reactions, and L-PHA binds to tri- and tetra-antennary oligosaccharides produced by GnTV-catalyzed reactions (Merkle and Cummings, 1987). These results show that tetracycline-regulated overexpression of glycosyltransferases is an effective way to manipulate the glycoform distributions of glycoproteins. For each glycosyltransferase, a clone with high expression in the absence of tetracycline, but with undetectable expression with tetracycline (clone 6, Fig. 3A, CHO-tet-GnTV, and clone 4, Fig. 3B, CHO-tet-GnTIII) was selected for further work.

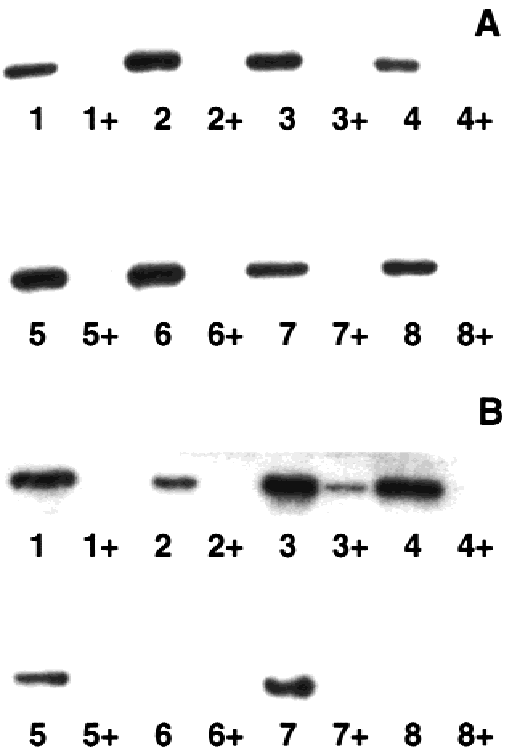

Figure 3. Screening of CHO clones for stable, tetracycline-regulated expression of GnTV (A) or myc-tagged GnTIII (B) glycosyltransferases by western blot analysis. CHO-tTA cells were co-transfected with a vector for expression of puromycin resistance (pPUR) and either pUHD10-3-GnTV (A) or pUDH10-3-GnTIIIm (B) and stable CHO clones were selected for resistance to puromycin (7.5 μg/mL), in the presence of tetracycline (2 μg/mL). Eight clones (1–8) for each glycosyltransferase were cultured for 48 h in the absence or presence (+) of tetracycline (2 μg/mL) and analyzed by western blot using either an anti-GnTV antibody (A) or an anti-myc (9E10) antibody (B).

Inhibition of Growth Due to Glycosyltransferase Overexpression

During screening of GnTIII- and GnTV-expressing clones in the absence of tetracycline, approximately half of each set of clones showed a strong inhibition of growth. The extent of growth-inhibition varied among clones, and comparison with expression levels estimated from western blot analysis (Fig. 3) suggested a correlation between the degree of growth-inhibition and glycosyltransferase overexpression. This correlation was firmly established by growing the final clones, CHO-tet-GnTIII and CHO-tet-GnTV, in different concentrations of tetracycline (Fig. 5). A strong inhibition of growth was evident after 2 days of culture at low levels of tetracycline (Fig. 6). Growth-inhibited cells displayed a small, rounded morphology instead of the typical extended shape of adherent CHO cells. After a few days, significant cell death was apparent from the morphology of the growth-inhibited cells.

Growth-inhibition due to glycosyltransferase overexpression has not hitherto been reported in the literature, probably

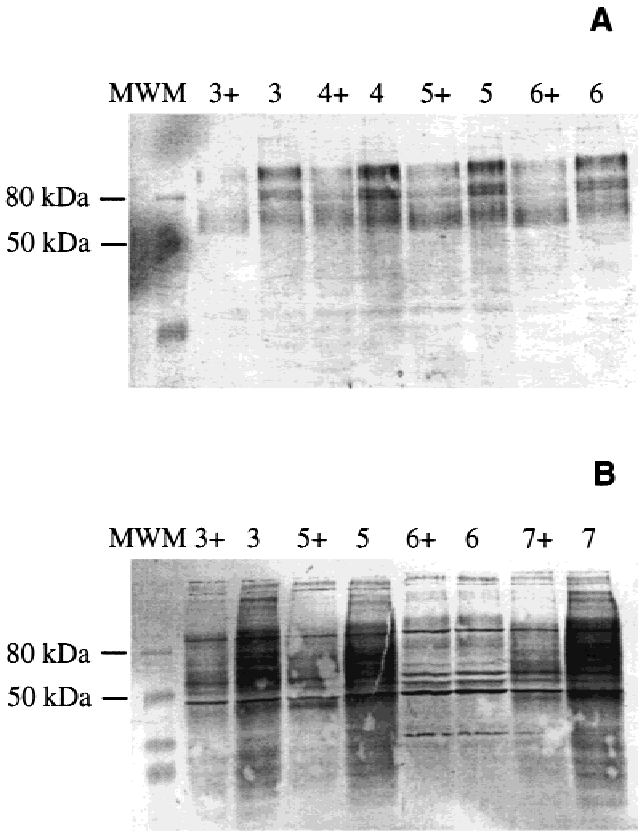

Figure 4. Verification of activity of heterologous GnTV (A) and GnTIII (B) glycosyltransferase in vivo by lectin blot analysis. Cellular glycoproteins from various stable clones (numbered as in Fig. 4), cultured in the absence or presence (+) of tetracycline (2 μg/mL), were resolved by SDS–PAGE, blotted to a membrane, and probed with either L-PHA (A) or E-PHA (B) lectins. These lectins bind with higher affinity to the oligosaccharide products of reactions catalyzed by GnTV and GnTIII, respectively, than to the oligosaccharide substrates of these reactions. A molecular weight marker (MWM) was run in parallel. A comparison of lectin blots in A and B indicates a broader range of substrates, among the endogenous CHO cell glycoproteins, for GnTIII (B) than for GnTV (A).

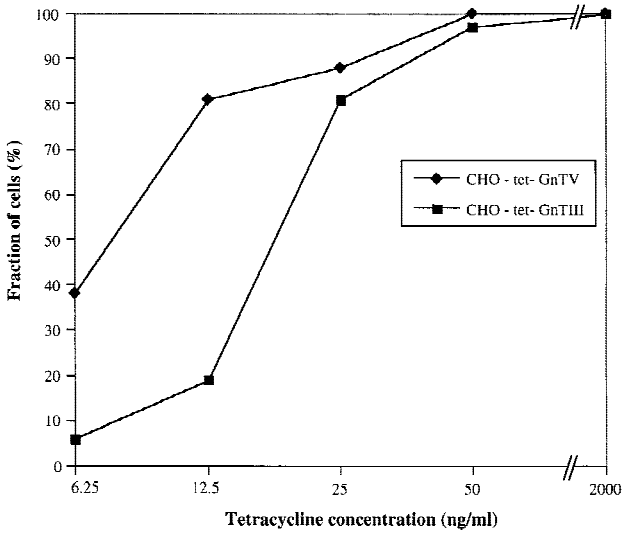

Figure 5. Inhibition of cell growth at different levels of glycosyltransferase overexpression. CHO-tet-GnTIII and CHO-tet-GnTV cells were seeded to 5–10% confluency and cultured in the presence of different levels of tetracycline. When the cultures grown at 2000 ng/mL of tetracycline reached confluency, cells from all the cultures were counted. The cell fraction is the ratio of the number of cells grown at a particular tetracycline concentration to the number of cells from the culture at 2000 ng/mL of tetracycline.

due to the widespread use of constitutive promoters. Those clones giving constitutive expression of a glycosyltransferase at growth-inhibiting levels would be lost during the selection-cloning procedure. This was avoided here by keeping tetracycline in the medium, i.e., by maintaining basal expression levels, throughout cloning. In addition, prior to selection, the frequency of clones capable of expressing glycosyltransferases to growth-inhibiting levels would be expected to be lower when using traditional mammalian vectors based on the constitutive hCMV promoter/enhancer. This is due to the fact that, for any given gene, the pUHD10-3 vector gives significantly higher expression levels than constitutive hCMV promoter/enhancer-based vectors, when transfected into CHO cell lines selected for high constitutive levels of tTA, as observed by others (Yin et al., 1996) and us (data not shown).

Inhibition of cell growth could be due to a direct effect of overexpression of membrane-anchored, Golgi-resident glycosyltransferases independent of their in vivo catalytic activity, for example via misfolding in the endoplasmic reticulum (ER) causing saturation of elements which assist protein folding in the ER. This could possibly affect the folding and secretion of other essential cellular proteins. Alternatively, inhibition of growth could be related to increased in vivo activity of the glycosyltransferase leading to a change of the glycosylation pattern, in a function-disrupting fashion, of a set of endogenous glycoproteins necessary for growth under standard in vitro culture conditions.

Independent of the underlying mechanism, the growth-inhibition effect has two consequences for engineering the glycosylation of animal cells. First, it implies that co-transfection of constitutive glycosyltransferase expression vectors together with vectors for the target glycoprotein product is a poor strategy. Other ways of linking expression of these two classes of proteins, for example through the use of multiple constitutive promoters of similar strength or use of multicistronic, constitutive expression vectors, should also be avoided. In these cases, clones with very high, constitutive expression of the target glycoprotein, a prerequisite for an economical bioprocess, would also have high expression of the glycosyltransferase and would be eliminated during the selection process. Linked, inducible expression could also be problematic for industrial bioprocesses, since the viability of the growth-arrested cells would be compromised by the overexpression of the glycosyltransferase. However, this configuration might be desirable if combined with expression of a cytostatic gene, resulting in simultaneous proliferation arrest in a productive configuration (Fussenegger et al., 1998) synchronized with remodelling of glycosylation machinery and product expression.

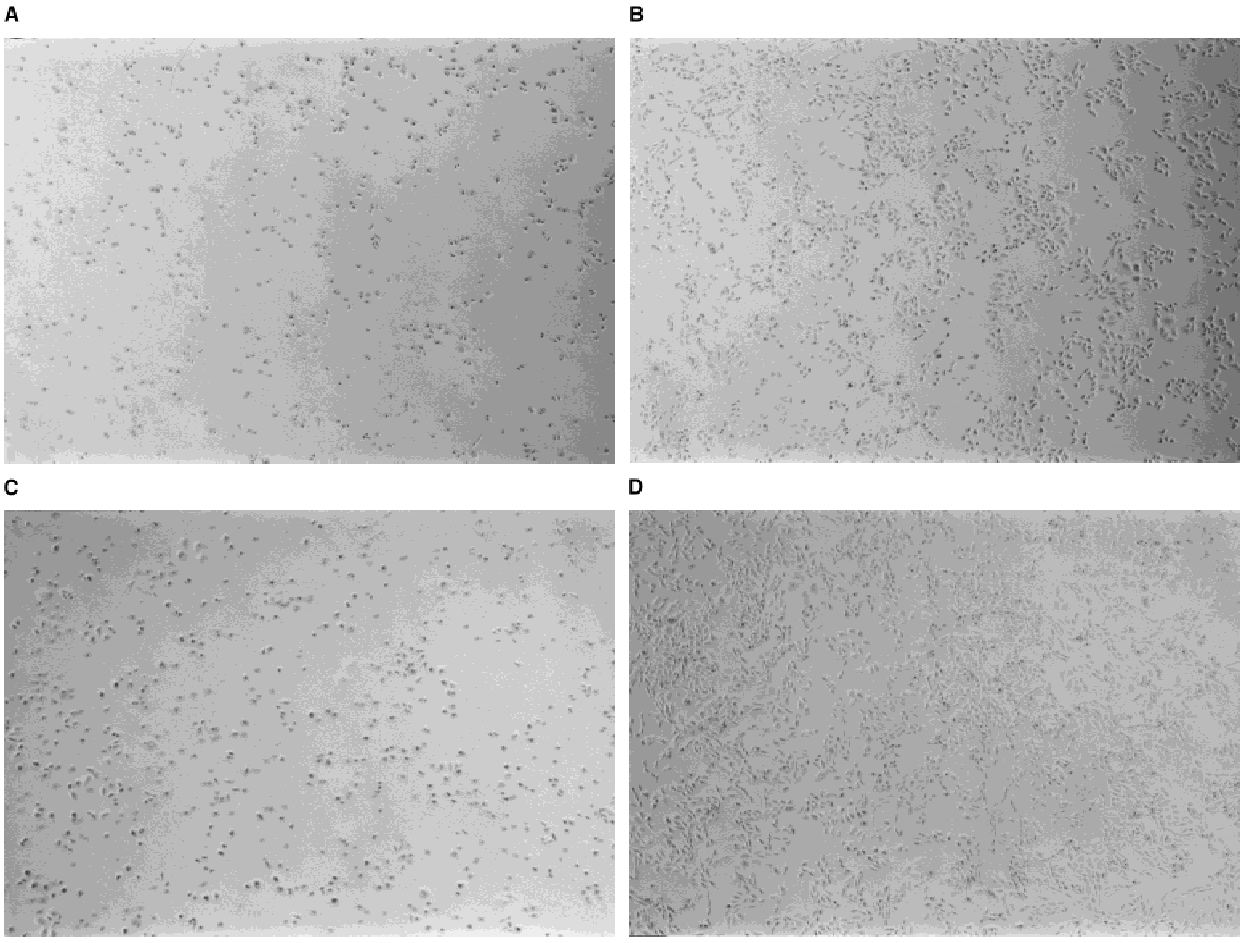

Figure 6. Inhibition of cell growth upon glycosyltransferase overexpression. CHO-tet-GnTIII cells (A and B) and CHO-tet-GnTV (C and D) were seeded to 5–10% confluency and cultured in the absence (A and C) or presence (B and D) of tetracycline (50 ng/mL). Cultures were photographed 45 h after seeding.

The second consequence of the growth inhibition effect imposition is an upper limit on glycosyltransferase overexpression for glycosylation engineering approaches. Clearly, the conversions of many glycosyltransferase-catalyzed reactions in the cell, at the endogenous levels of glycosyltransferases, are very high for several glycosylation sites. However, glycosylation sites where the oligosaccharides are somewhat inaccesible or are stabilized in unfavorable conformations for specific glycosyltranferases also exist. For example, it has been observed that addition of bisecting GlcNAc to the oligosaccharides attached to the Fc region is more restricted than to oligosaccharides located on the variable regions of human IgG antibodies (Savvidou et al., 1984). Glycosylation engineering of these restricted sites could be affected by such a limit on glycosyltransferase expression. Induced expression of a glycosyltransferase after cells have grown to high density, before or concomitant with product expression, should allow significantly higher cloned glycosyltransferase activities than can be accommodated in cells which must grow to high density while constitutively expressing a cloned glycosyltransferase.

Finally, it would be useful to determine if the growth-inhibiting effect of glycosyltransferase expression is additive. If so, the limit could become more important for glycosylation engineering approaches involving several glycosyltransferases.

This research was supported by the Swiss Priority Program in Biotechnology (SPP BioTech). In addition, the authors thank Dr. Naouyuki Taniguchi for providing the GnTIII and GnTV cDNAs, Dr. Michael Pierce for an anti-GnTV antibody, Dr. John Robinson for 9E10 mAb, Dr. Herman Bujard for plasmid vectors of the tet-regulated system, and Heidi Ernst for automated DNA sequencing.

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