The Signal for Golgi Retention of Bovine β1,4-Galactosyltransferase Is in the Transmembrane Domain*

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The expression and localization of bovine β1,4-galactosyltransferase (Gal T) has been studied in mammalian cells transfected with Gal T cDNA constructs, and the role of the amino-terminal domains of Gal T in Golgi localization examined. Here we demonstrate that the transmembrane (signal/anchor) domain of bovine Gal T contains a positive Golgi retention signal. Bovine Gal T was characterized in transfected cells with anti-bovine Gal T antibodies, affinity-purified from a rabbit antiserum using a bacterial recombinant fusion protein. These affinity-purified antibodies recognized native bovine Gal T and showed minimum cross-reactivity with Gal T from non-bovine sources. Bovine Gal T cDNA was expressed, as active enzyme, transiently in COS-1 cells and stably in murine L cells, and the product was shown to be localized to the Golgi complex by immunofluorescence using the polypeptide-specific antibodies. A low level of surface bovine Gal T was also detected in the transfected L cells by flow cytometry. The removal of 18 of the 24 amino acids from the cytoplasmic domain of bovine Gal T did not alter the Golgi localization of the product transiently expressed in COS-1 cells or stably expressed in L cells. Both the full-length bovine Gal T and the cytoplasmic domain deletion mutant were N-glycosylated in the transfected L cells, indicating both proteins have the correct Nα/Cα, membrane orientation. Deletion of both the cytoplasmic and signal/anchor domains of bovine Gal T and incorporation of a cleavable signal sequence resulted in a truncated soluble bovine Gal T that was rapidly secreted (within 1 h) from transfected COS-1 cells. Replacement of the signal/anchor domain of bovine Gal T with the signal/anchor domain of the human transferrin receptor resulted in the transport of the hybrid molecule to the cell surface of transfected COS-1 cells. Furthermore, a hybrid construct containing the signal/anchor domain of Gal T with ovalbumin was efficiently retained in the Golgi complex, whereas ovalbumin anchored to the membrane by the transferrin receptor signal/anchor was expressed at the cell surface of transfected COS-1 cells. Overall, these studies show that the hydrophobic, signal/anchor domain of Gal T is both necessary and sufficient for Golgi localization.

The terminal carbohydrate sequences of glycoproteins perform a diverse range of biological roles, for example molecular recognition events such as targeting of lysosomal hydrolases (Kornfeld, 1987) and cell adhesion (Brandley et al., 1990; Springer and Laskey, 1991). The biosynthesis of these carbohydrate sequences is catalyzed by specific glycosyltransferases located within the Golgi complex through a series of elongation reactions (Kornfeld and Kornfeld, 1985; Schachter, 1986; Gleeson, 1988). UDP-galactose:N-acetylgalosamine β1,4-galactosyltransferase (Gal T) is involved in the biosynthesis of Galβ1-4GlcNAc sequences, found in glycoproteins and glycolipids, by catalyzing the transfer of galactose, from UDP-Gal, to the terminal non-reducing GlcNAc residues of oligosaccharide chains (Beyer et al., 1981; Strous, 1986). The cDNAs encoding Gal T have been isolated from human (Masri et al., 1988), bovine (Shaper et al., 1986; Narimatsu et al., 1986; D’Agostaro et al., 1989), and murine (Nakazawa et al., 1988; Shaper et al., 1988) sources. The deduced amino acid sequences of Gal T are highly conserved across species, for example the bovine and murine Gal T sequences are 86% similar (D’Agostaro et al., 1989). Like other glycosyltransferases, Gal T is predicted to have an Nα/Cα orientation (type II membrane protein) (Paulson and Colley, 1989), with a 24-amino acid amino-terminal cytoplasmic tail, a 20-amino acid signal/anchor domain, a luminal exposed stem domain of 61 amino acids, and a large 42-kDa luminal catalytic domain (D’Agostaro et al., 1989). Two transcriptional initiation sites have been reported for both murine and bovine Gal T and, further, two bovine Gal T mRNA transcripts of different lengths have been identified. Both transcripts give rise to membrane-bound products, with the shorter form containing an 11-amino acid amino-terminal cytoplasmic tail as compared with 24 amino acids for the longer transcript (Shaper et al., 1988; Russo et al., 1990). Gal T has been localized by immunoelectron microscopy to the trans-cisternae of the Golgi (Roth and Berger, 1982; Suganuma et al., 1991). In addition to a trans-Golgi localization, there have been a number of reports of the cell surface expression of Gal T (Shur, 1982; Sato et al., 1984; Shaper et al., 1985; Roth et al., 1985a; Marchese et al., 1988). A number of these studies have involved immunochemical approaches using antibodies raised to the native Gal T glycoprotein. However, as it has been demonstrated that affinity-purified antibodies to a human Gal T recognized blood group.

1 The abbreviations used are: Gal T, β1,4-galactosyltransferase; PBS, phosphate-buffered saline; MDMK, Madin-Darby bovine kidney; IPTG, isopropyl-β-D-thiogalactopyranoside; FACS, fluorescence-activated cell sorter; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TTR, human transferrin receptor; Ova, ovalbumin; PCR, polymerase chain reaction; NBD, 1-(N-methyl-N-(7-nitrobenz-2-oxa-1,3-diazole-4-yl)); DMEM, Dulbecco's modified Eagle's medium; bp, base pair(s).

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specific carbohydrate structures on the enzyme, the reactivity with cell surface components may be due to cross-reactivity of the anti-Gal T antibodies with cell surface carbohydrate epitopes (Childs et al., 1986; Feizi et al., 1987). Clearly, antibodies specific for the polypeptide of Gal T are required to clarify this issue.

In addition to the membrane-associated Gal T, active soluble forms of the enzyme exist in body fluids such as milk, colostrum, and serum (for review see Strous, 1986). In mammary tissue, the specificity of Gal T is modified to a lactase synthetase, by association with α-lactalbumin, which is responsible for the synthesis of the β-lactase lactose (Trayer and Hill, 1971). This soluble form of the enzyme represents the luminal, catalytic domain of the membrane enzyme and is thought to be derived from the membrane-bound form by proteolytic cleavage. Bioinformatic studies of human Gal T in cultured cells have indeed revealed that Gal T has an intracellular half-time of about 19 h, after which it is released into the culture medium (Strous and Berger, 1982).

The signals responsible for the localization of resident GalT proteins have not been identified. Based on the current model of bulk flow from the endoplasmic reticulum to the cell surface, proteins resident in the Golgi should be expected to contain a Golgi retention signal (Pfeffer and Rothman, 1987). Such retention signals have been found to reside on resident soluble proteins (Munro and Pelham, 1987; Pelham, 1990) and resident membrane proteins (Gabathuler and Kvist, 1990; Jackson et al., 1990; Shin et al., 1991) of the endoplasmic reticulum. The cDNAs for a number of membrane glycosyltransferases that act at the late Golgi stage have been cloned and sequenced (Paulson and Colley, 1988; Larsen et al., 1989, 1990; Joziasse et al., 1989; Yamamoto et al., 1990). Comparison of the primary amino acid sequences of these cloned glycosyltransferases have not revealed an obvious potential Golgi retention signal. As the secreted form of Gal T found in body fluids lacks the stem, signal/anchor, and cytoplasmic domains, it is likely that the signals responsible for Golgi localization are contained within these three domains. Indeed, α2,6-sialyltransferase has been converted from a membrane-bound enzyme into a secreted form by the deletion of these three domains (Colley et al., 1989a).

Here, we report the generation of antibodies specific for the polypeptide of bovine Gal T. We have expressed bovine Gal T in transfected mammalian cells and have demonstrated, using the bovine-specific anti-Gal T antibodies, that the product is localized in the bovine COS-1 or murine L cells. In addition, from the localization of deletion mutants of Gal T and of hybrid molecules, we have demonstrated that the signal/anchor domain of Gal T contains a positive signal responsible for Golgi localization.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Cell lines were maintained in exponential growth as monolayers in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum, 2 mm glutamine, 100 units/ml penicillin, and 0.1% (w/v) streptomycin (complete DMEM). Madin-Darby bovine kidney (MDBK) epithelial cells (Commonwealth Serum Laboratories, Australia) were maintained in complete medium supplemented with nonessential amino acids. Mouse L fibroblasts (tk +) were obtained from C. Bird (Department of Pathology and Immunology, Monash Medical School).

**cDNA Construction**—The full-length cDNA for bovine Gal T was generated from two overlapping cDNAs isolated by D’Agostaro et al., (1989), namely pGPT/P8 (1-11039 bp) and pGTP/PA (947-1728 bp). As there was not a unique restriction site in the overlapping region, the two cDNA inserts were subcloned sequentially into the EcoRI site of pGEM 9zF(-) (Promega Corp.) such that the inserts were oriented appropriately. This plasmid was then digested with Stul and KpnI to excise the 532-bp Stul/KpnI fragment. The remaining plasmid fragment was then isolated and ligated with the 110-bp Stul/BsalI fragment isolated from pGTP/P8 and the 350-bp BsalI/KpnI fragment isolated from pGPT/PA in a three-fragment ligation reaction. The final plasmid, pGEM-GalT, contained the complete coding region for bovine Gal T (Fig. 1d).

The full-length cDNA (1728 bp) for bovine Gal T was subcloned into the EcoRI site of pSVTgpt, with the 110-bp cDNA inserts were subcloned sequentially into the EcoRI site of pGEM 9zF(-) containing the selectable marker aminopterin resistance, and the vector containing the selectable marker neomycin resistance (Mulligan and Berg, 1981). This vector contains the SV40 origin of replication and early region promoter, followed by a multiple restriction site and an SV40 polyadenylation signal.

A construct lacking the cytoplasmic tail and signal/anchor domain of Gal T was prepared by digestion of pGEM-GalT with Nael, and the fragment of 1844 bp was sequenced (see Fig. 1a). The 5' Nael site, located at bp 312 of the Gal T cDNA, corresponds to the start of the stem domain (residue 43), and the 3' Nael site is 533 bp downstream of the EcoRI cloning site (residue 1050). This 1508-bp fragment was blunt-end ligated into the unique Smal site of the expression vector pSHT.3 The Smal site is at the 3' end of the hemagglutinin signal peptide sequence in this vector, and this ligation resulted in an in-frame construct designated pSHT-GalT(-)/T,-S/A (Fig. 1d).

A construct containing only the catalytic domain of Gal T was prepared as follows. pGEM-GalT was digested with Mulu and EcoRI, and the 5' overhangs were filled in with the Klenow fragment of DNA polymerase I and dNTPs. The 1240-bp fragment was isolated from an agarose gel and purified (GeneClean, BIO 101, La Jolla, CA). The Mulu site of Gal T cDNA corresponds to amino acid 104, and this 1196-bp fragment encodes the 42-kDa soluble form of bovine milk Gal T (D’Agostaro et al., 1989) with three additional amino acids from the stem region (residues 104-107). This 1240-bp fragment was blunt end-ligated into pSHT previously digested with SpeI and treated with the Klenow fragment of DNA polymerase I and dNTPs. The unique SpeI site of pSHT is at the 3' end of the hemagglutinin signal peptide sequence in this vector, and this ligation resulted in the construct designated pSHT-GalT(T,-S/A,-Tm) (Fig. 1e).

The cDNA encoding the human transferrin receptor (TTR) (a gift of L. Kuhn) (McClelland et al., 1984) was cloned into the EcoRI and XbaI sites of pSVT-7 and is referred to as pSVT-TTR. A construct with the Gal T signal/anchor domain replaced by the signal/anchor domain of the human transferrin receptor, designated pSVTgpt-GalT/TTR(S/A) (Fig. 1f), was prepared as follows. Initially, a 110-bp polymerase chain reaction (PCR) product, containing the signal/anchor domain of the transferrin receptor, was generated using pSHT as template. Subsequently, this PCR fragment was blunt-end ligated into the unique NdeI site of pSVT-TTR (a gift of L. Kuhn). This cloned cDNA represented the 5' and 3' sequences of the human transferrin receptor signal/anchor domain and extended by Gal T sequences that included restriction sites to allow substitution of the signal/anchor domain of Gal T for the PCR product. The 5' oligonucleotide primer 5'-CCCTGCACGGCGCCCTGCTCCTGATCTGGAAAAGGTATCTGCAGCGGGCATAGCCCAAGTAGCCAAT-3' represents the first 18 nucleotides of the signal/anchor domain of the transferrin receptor (underlined), with 20 nucleotides of Gal T corresponding to the cytoplasmic domain (amino acid sequence 19-24), which also includes a SalI site (Fig. 1a). The 3' oligonucleotide primer 5'-CTCGGCCGGCATAGCCCAAGTAGCCAATGATTCTCCGTCGCAGGGCCCTGCCGCTGTAGTGGAAGTATCTGCAGCGGGCATAGCCCAAGTAGCCAAT-3' represents the last 18 nucleotides of the signal/anchor domain of the human transferrin receptor (underlined) and the first 10 nucleotides of the intraluminal domain of Gal T, which includes an ECM site. The reaction was carried out in 10 mM Tris-Cl, pH 8.5, containing 50 mM KCl, 1.5 mM MgCl2, 0.01% (w/v) gelatin, 4 mm dNTPs, 100 pmol of each oligonucleotide, 0.5 units of Taq polymerase (Promega Corp.) such that the two primers were oriented appropriately. This plasmid was then digested with SalI and XbaI to excise the 580-bp SalI/XbaI fragment. The resulting plasmid fragment was then isolated and ligated with the 110-bp SalI/XbaI fragment isolated from pGTP/P8 and the 350-bp XbaI/SalI fragment isolated from pGPT/PA in a three-fragment ligation reaction. The final plasmid, pGEM-GalT, contained the complete coding region for bovine Gal T (Fig. 1a).

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Here, we report the generation of antibodies specific for the polypeptide of bovine Gal T. We have expressed bovine Gal T in transfected mammalian cells and have demonstrated, using the bovine-specific anti-Gal T antibodies, that the product is localized in the bovine COS-1 or murine L cells. In addition, from the localization of deletion mutants of Gal T and of hybrid molecules, we have demonstrated that the signal/anchor domain of Gal T contains a positive signal responsible for Golgi localization.
FIG. 1. Construction of plasmids.

(a) pGEM-GalT

Thick line, the vector pGEM 9zf(-); open box, multiple cloning site of pGEM 9zf(-); closed box, the open reading frame of bovine Gal T cDNA; thin lines, untranslated regions of the cDNA. The regions of Gal T cDNA correspond to the cytoplasmic domain (CT), the signal/anchor domain (S/A), the intraluminal stem (Stem), and the 42-kDa secreted form of Gal T (Catalytic Domain). Restriction endonuclease sites are indicated as follows: B, BsaI; E, EcoRI; K, KspI; Kp, KpnI; M, MluI; N, NaeI; P, PvuII; Ps, PstI; S, StuI. More than one BsaII site is present in the plasmid, and the site referred to in the text is indicated by the asterisk.

(b) pSHT-GalT

Structure of plasmids containing full-length Gal T cDNA and deletion mutants of Gal T. Thick line, the expression vector consisting of the SV40 early promoter (VE) and the SV40 polyadenylation signal (POLYA). The pSHT expression vector includes the sequence encoding the leader peptide of influenza hemagglutinin (HA). The numbers below the amino acid sequence refer to the sequence of the polypeptide encoded by the mutant construct. The boxed sequences indicate the Gal T sequence, and the numbers above the boxes refer to the corresponding amino acid sequence of full-length Gal T. j-h, structure of hybrid constructs. Closed boxes, bovine Gal T cDNA; open boxes, human transferrin receptor cDNA; shaded boxes, ovalbumin cDNA. The amino acid sequences of the junction regions are indicated as above.

sample was analyzed on a 2% agarose gel, and a single band of 110 bp was detected. The PCR product was then incubated with 50 µg/ml of proteinase K in 0.5% SDS at 37 °C for 30 min (Crowe et al., 1991), and the sample was heat-inactivated at 68 °C for 10 min. The sample was extracted with phenol and the DNA precipitated with ethanol. To fill in overhangs and phosphorylate 5' ends of the PCR product, an incubation was carried out in 50 mM Tris-HCl, pH 7.6, containing 10 mM MgCl₂, 10 mM dithiothreitol, 50 µg/ml bovine serum albumin, 1 mM dNTPs, 1 mM ATP, 12.5 units of T7 DNA polymerase, and 20 units of T4 polynucleotide kinase for 30 min at 37 °C. After the incubation, the sample was heat-inactivated, and the DNA fragments were then self-ligated by addition of polyethylene
glycol to 5% and 3 units of T4 DNA ligase followed by a 16-h incubation at 14 °C. After the incubation, the sample was extracted with phenol and the DNA was ethanol-precipitated, digested with PstI, and then subcloned into pSVTgpt-GalT previously digested with PstI to release a 1500-bp fragment. Clones containing the PCR product in the correct orientation were digested with EcoRI and with PstI to release a 1500-bp fragment. Clones containing the first 44 codons of Gal T cDNA, was isolated from an agarose gel and purified. A 1500-bp PstI fragment from pGEM-GalT (Fig. 1a), encoding the carboxyl-terminal 350 amino acids of Gal T, was recovered from an agarose gel using GeneClean (BIO 101) and ligated into the bacterial expression vector pGEX-3X, previously digested with EcoRI and dephosphorylated with calf intestinal phosphatase. Competent Escherichia coli DH1 cells were then transformed with the recombinant DNA (pGEX-GalT). The transformed bacteria were incubated overnight at 37 °C in L-broth, and then diluted 1:10 in L-broth and grown for a further 1 h at 37 °C. The synthesis of the fusion protein was then induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and a further incubation for 3 h at 37 °C. After the incubation, the bacteria were centrifuged and dissolved in SDS sample electrophoresis buffer (Laemmli, 1970), and the proteins were separated by SDS-PAGE and either stained with Coomassie Blue R-250 or electrophoretically transferred to nitrocellulose for immunoblotting.

Preparation of a Bovine Gal T Fusion Protein Affinity Matrix—pGEX-GalT-transformed bacteria were harvested from 1000-mL cultures incubated with 1 mM IPTG for 4 to 6 h at 37 °C (Smith and Johnson, 1988). The bacteria were pelleted, resuspended in 5 mM Tris, pH 8.0, and subjected to three freeze-thaw cycles by snap freezing on dry ice followed by thawing at 37 °C. The suspension was sonicated on ice twice for 30 s and then incubated on ice for 30 min. The extract was then centrifuged at 10,000 × g for 5 min, and the pellet, containing the majority of the fusion protein, was further incubated in 10 mL of 0.075 M sodium pyrophosphate, pH 8.0, containing 0.5% SDS, for 20 min on ice. The 0.5% SDS extract was centrifuged at 10,000 × g for 5 min, and the supernatant was analyzed by SDS-PAGE. The fusion protein constituted approximately 30% of the protein present in this SDS solubilized fraction. This fusion protein-enriched fraction was fractionated by preparative SDSPAGE and purified antibodies to bovine Gal T (Ulrich et al., 1986) were used at a protein concentration equivalent to that of the affinity-purified antibodies in all experiments. Affinity Purification of Anti-bovine Gal T Antibodies to Polypeptide Epitopes—The Gal T fusion protein-Sepharose 4B column was pre-eluted with 100 mM glycine, pH 2.6, and equilibrated with 10 mM Tris, pH 7.5. One mL of anti-bovine Gal T antiserum (from the bleed at day 65), diluted 1:5 with 10 mM Tris, pH 7.5, was then applied at 4 °C. The sample was passed through the column three times and then the column was washed with 10 bed volumes of 10 mM Tris, pH 7.5, containing 0.5 M NaCl. Bound antibodies were eluted from the column with 100 mM glycine HCl, pH 2.6, and the fractions were immediately neutralized and then dialyzed against PBS overnight. The affinity-purified antibodies, at a concentration of 0.2 mg/mL, were stored at 4 °C or −20 °C with 0.02% sodium azide. Affinity-purified antibodies were reacted with 40% ammonium sulfate precipitate of preimmune serum. This immunoglobulin fraction was used at a protein concentration equivalent to that of the affinity-purified antibodies in all experiments.

Transfections—Approximately 105 COS-1 cells/75-cm² tissue culture flask were transfected with 2.5 μg of DNA using the DEAE-dextran procedure with a chloroquine incubation (Sambrook et al., 1989), L (+) cells were transfected by the calcium phosphate precipitate method, followed by a glycerol shock (Sambrook et al., 1989). Transformants containing the dominant selectable marker ipt, were selected in medium containing 250 μg/ml xanthine, 6 μg/ml mycophenolic acid, and 500 μM hypoxanthine. 0.4 μM anti-nterin, and 16 μM thymidine. Following drug selection, selectable marker-resistant clones were isolated by limiting dilution in 96-well trays containing selection medium. Cell growth was monitored routinely.

Production of Rabbit Anti-bovine Gal T Antiserum—Commercially available bovine milk Gal T (Sigma) has been reported to be contaminated with bovine immunoglobulin (Ulrich et al., 1986), a result confirmed with the batches used in these experiments. Therefore, bovine milk Gal T (Sigma, lot 99F-8225) was passed through a 2-mL affinity column of sheep anti-bovine immunoglobulin (Silenus Laboratories, Australia) coupled to Sepharose 4B (Pharmacia, Uppsala) at a concentration of 3 mg/ml swollen gel, following the manufacturer's instructions.

Golgi Retention Signal of Galactosyltransferase was tested for binding to MDBK cells by indirect immunofluorescence. Serum was stored at −70 °C after addition of 0.02% sodium azide.

**Recombinant Bovine Gal T Fusion Protein—** A fusion protein containing the first 44 codons of the intracellular domain of bovine Gal T at the carboxyl terminus of glutathione S-transferase, using the plasmid expression vector pGEX (Smith and Johnson, 1988), as follows. A 1500-bp PstI fragment from pGEM-GalT (Fig. 1a), encoding the carboxyl-terminal 350 amino acids of Gal T, was recovered from an agarose gel using GeneClean (BIO 101) and ligated into the bacterial expression vector pGEX-3X, previously digested with EcoRI and dephosphorylated with calf intestinal phosphatase. Competent Escherichia coli DH1 cells were then transformed with the recombinant DNA (pGEX-GalT). The transformed bacteria were incubated overnight at 37 °C in L-broth, and then diluted 1:10 in L-broth and grown for a further 1 h at 37 °C. The synthesis of the fusion protein was then induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and a further incubation for 3 h at 37 °C. After the incubation, the bacteria were centrifuged and dissolved in SDS sample electrophoresis buffer (Laemmli, 1970), and the proteins were separated by SDS-PAGE and either stained with Coomassie Blue R-250 or electrophoretically transferred to nitrocellulose for immunoblotting.

Fusion protein constituted approximately 30% of the protein present in this SDS solubilized fraction. This fusion protein-enriched fraction was fractionated by preparative SDSPAGE and purified antibodies to bovine Gal T (Ulrich et al., 1986) were used at a protein concentration equivalent to that of the affinity-purified antibodies in all experiments.
with the primary antibody. The affinity-purified anti-bovine Gal T antibodies (50 μl) were used at a protein concentration of 2 μg/ml (diluted from stock in PBS). The preimmune immunoglobulin fraction was diluted to the same protein concentration. Human transferrin receptor antibodies were preincubated with the purified bovine monoclonal antibody OKT9 (IgG1) (Schneider et al., 1982) prepared from hybridoma culture supernatants. Ovalbumin hybrid proteins were detected with rabbit anti-ovalbumin IgG (Organon Teknika-Cappel, Turnhout, Belgium) diluted 1:200 in PBS (final IgG concentration 20 μg/ml). Monolayers were washed in PBS and then incubated for 45 min with fluorescent isothiocyanate-conjugated sheep anti-rabbit immunoglobulin or fluorescein isothiocyanate-conjugated sheep anti-mouse immunoglobulin (1:100 dilution) (Silenus Laboratories, Australia), washed again in PBS, and mounted in PBS/glycerol (9:1) containing 1 mg/ml p-phenylenediamine. Monolayers were examined with a Zeiss fluorescence microscope.

The Golgi complex of unfixed cells was stained using the fluorescent sphingolipid vital stain C<sub>6</sub>-NBD-ceramide (Molecular Probes, Inc.) complexed to bovine serum albumin as described by Lipsky and Pagano (1985).

Drug Treatment—Stock solutions of 10 mM nodocazole (Sigma) in dimethyl sulfoxide and 5 mg/ml brefeldin A (a gift from Sandoz Co., Turnhout, Belgium) in methanol were stored at -20 °C. Cells were grown on 12 well slides, the medium overlaying the cells was replaced with fresh medium (50 μl) containing either 10 μM of nodocazole or 5 μg/ml brefeldin A, and the monolayers were incubated for 45 min at 37 °C. After drug treatment, the cell monolayers were fixed and processed for immunofluorescence. Control experiments were carried out by incubating cells for the equivalent time in drug-free medium containing either 0.1% dimethyl sulfoxide or 0.1% methanol. No effect on the staining pattern was observed in these control experiments.

Flow Cytometry—Subconfluent monolayers of cells, preincubated for 24 h with Opti MEM I media (GIBCO-BRL) (serum-free) to eliminate possible contamination of cells by bovine Gal T derived from the fetal calf serum, were washed with saline and harvested with 50 mM EDTA in PBS. For detection of surface-bound Gal T, cell suspensions (2 × 10<sup>6</sup> cells) were washed twice with DMEM (serum-free), preincubated with PBS containing 20 mg/ml bovine serum albumin (Fraction V, Sigma) for 30 min. The cells were then incubated with affinity-purified anti-Gal T antibodies or with preimmune immunoglobulin (200 μg of 2 μg/ml, diluted in PBS) for 60 min on ice. Cells were washed twice in PBS and then incubated for 60 min on ice with fluorescein isothiocyanate-conjugated sheep anti-rabbit immunoglobulin (Silenus Laboratories, Australia) diluted 1:100 in PBS and then were finally washed in PBS. Stained cells were analyzed on a FACS II (Becton Dickinson) after addition of propidium iodide (Sigma), using Cell Quest for analysis.

For analysis of both intracellular and surface-localized proteins, the cell suspensions were initially washed twice with PBS, fixed with 4% formaldehyde in PBS at room temperature for 20 min, washed again in DMEM (serum-free), and permeabilized with 1% Triton X-100, PBS for 20 min on ice. After permeabilization, the cell suspensions were incubated with 10 μM of a 10% suspension of fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulin, incubated on ice for 10 min, and removed by centrifugation. The supernatants were assayed for Gal T activity. Aliquots of cell extract (all containing the same Gal T activity) were incubated with either 40 μl of affinity-purified antibody or preimmune serum, diluted in PBS, for 60 min on ice. The immune complexes were then precipitated with 50 μl of a 10% suspension of Protein A-Sepharose 4B as described above. The protein A-Sepharose 4B beads were then washed using the buffers described by Owen and co-workers (1985) and eluted with 0.1 M glycine, pH 2.5. The eluates were analyzed for Gal T activity using 20 mM glucose as acceptor and a-lactalbumin (Sigma) added to a final concentration of 1 mg/ml.

For Gal T activity of cultured cells, subconfluent cell monolayers were harvested in PBS by scraping with a rubber policeman, and the cells were pelleted by centrifugation and extracted in 1% Triton X-100, PBS for 30 min on ice. Insoluble material in the extract was removed by centrifugation at 15,000 × g for 15 min, and the supernatant was assayed for Gal T activity.

Absorption of Gal T Activity by Immunoprecipitation—Semiconfluent cell monolayers from two 75-cm<sup>2</sup> tissue culture flasks were harvested in PBS by scraping with a rubber policeman, and the cells were pelleted at 450 × g for 5 min at 4 °C. The cell pellet was extracted in 500 μl of 1% Triton X-100 in PBS on ice for 30 min, and nuclei and cell debris were removed by centrifugation. Pansorbin (200 μl of a 10% suspension; Calbiochem), prewashed three times in 50 mM Tris-Cl, pH 9.0, 0.6 M NaCl, 0.5% Triton X-100, was added to the cell extract, incubated on ice for 10 min, and removed by centrifugation. The supernatants were assayed for Gal T activity. Aliquots of cell extracts (all containing the same Gal T activity) were incubated with either 40 μl of affinity-purified antibody or preimmune serum, diluted in PBS, for 60 min on ice. The immune complexes were then precipitated with 50 μl of a 10% suspension of Pansorbin, followed by a 10-min incubation on ice and centrifugation at 15,000 × g for 5 min. Aliquots (50 μl) of the original extracts and final supernatants were then assayed for Gal T activity.

Biopsy Synthetic Radiolabeling of Cultured Cells and Immunoprecipitation—Subconfluent L cells in 94-mm (diameter) Petri dishes (Greiner, Labortechnik) were washed twice with sterile saline and then incubated in serum-free, methionine-free RPMI 1640 (Flow Laboratories, Australia) supplemented with 2 mM glutamine, at 37 °C for 45 min. The cells were radiolabeled with 1.5 ml of fresh medium containing 0.25 mCi of [35S]-methionine and -cysteine (Express 3'S-protein-labeling mix, Du Pont-New England Nuclear, Australia; specific activity of L-[35S]methionine, -1100 Ci/ml) and incubated at 37 °C for 3-4 h. After labeling, the cells were washed twice in sterile saline and then extracted in 1 ml of lysis buffer (PBS containing 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 0.01% sodium azide, 5 mM EDTA, 0.1 M sodium chloride, 1 μM phenylmethylsulfonyl fluoride, 1 mM leupeptin, 1 mM aprotinin, 1 mM peptatin) for 30 min on ice. Nuclei and cell debris were removed by centrifugation at 10,000 × g for 15 min at 4 °C. The incorporation of [35S]methionine and -cysteine into cellular protein was measured by trichloroacetic acid precipitation and counting of the precipitate.

For immunoprecipitations, the [35S]-labeled cell extracts were initially preclreated by sequential additions of four aliquots of protein A-Sepharose 4B (Pharmacia, Sweden) (100 μl of a 10% suspension), each of which was incubated with rotation for 15 min at 4 °C and then removed by centrifugation. Preclreated extracts (500 μl) were then incubated with 0.1% affinity-purified antibody or preimmune serum, or the preimmune immunoglobulin, for 4 h on ice, and the immune complexes were collected by the addition of 100 μl of a 10% suspension of protein A-Sepharose 4B as described above. The protein A-Sepharose 4B beads were then washed using the buffers described by Owen et al. (1980), except that 10 mg/ml of bovine serum albumin was included in the first two buffers. The immune complexes were solubilized in electrophoresis sample buffer at 100 °C for 2 min and analyzed by SDS-PAGE (Laemmli, 1970) on a 10% polyacrylamide gel. The gels were treated with AmphiTuff (Amersham, United Kingdom) and fluorographed at -70 °C using Du Pont Lightning Plus intensifying screens and Fuji x-ray film.

For pulse-chase experiments, COS-1 cells were incubated in serum-free and methionine-free RPMI 1640 and radiolabeled as for the L cells approximately 48 h following transfection. The pulse-labeling was terminated after 30 min by removal of the medium, the cells were washed twice with complete DMEM, and fresh prewarmed complete DMEM was added for the duration of the chase period. At the end of each chase interval, dishes of labeled COS-1 cells were placed on ice, the chase medium was collected, and the cells were lysed as described above. Immunoprecipitations were carried out on the cell extracts as described above for the L cells. Equivalent amounts of labeled cell extracts and chase medium were measured at each time point.

Peptide N-Glycosidase F Digestion—Radiolabeled Gal T was im-
Preparation of Antibodies Specific for Bovine β1,4-Galactosyltransferase—To investigate the expression and localization of bovine Gal T in eukaryotic expression systems, antibodies specific for the polypeptide of the glycoprotein were required. Initially, synthetic peptides corresponding to the cytoplasmic tail of bovine Gal T and an insoluble bacterial fusion protein encoding the 147 carboxyl-terminal amino acids from the luminal domain of bovine Gal T were used as immunogens. However, both antigens failed to induce antibodies in rabbits capable of reacting with the native enzyme from cultured bovine cells. Therefore, an alternative strategy was adopted. Immunization of rabbits with a preparation containing soluble, glycosylated, active forms (approximately 42 and 48 kDa) of bovine milk Gal T produced an antiserum that bound to acetone-fixed and permeabilized MDBK epithelial cells (data not shown).

To purify antibodies to polypeptide epitopes of the bovine Gal T, a bacterial recombinant fusion protein was generated that consisted of 350 carboxyl-terminal amino acids of bovine Gal T contiguous with the carboxyl terminus of the 26-kDa glutathione-S-transferase. This fusion protein contains most of the luminal domain of bovine Gal T and includes the sequence of the soluble form of Gal T used as the immunogen. The resulting 64-kDa fusion protein (Fig. 2A; compare lanes 1 and 2) was insoluble in 1% Triton X-100 and was recovered in the Triton X-100-insoluble fraction. The Triton X-100-insoluble fraction was solubilized in 0.5% SDS, and this fraction, enriched for the fusion protein (Fig. 2A, lane 3), was then directly coupled to CNBr-activated Sepharose 4B. Anti-Gal T antibodies were purified from the rabbit antiserum by glutathione-S-transferase.

Immunofluorescence of acetone-fixed MDBK cells with the affinity-purified antibodies specifically absorbed Gal T activity from 1% Triton X-100 extracts of MDBK cells; therefore, the antibodies purified from the SDS-solubilized fusion protein recognized the enzyme in its native conformation. On the other hand, the anti-bovine Gal T antibodies, probably due to the distribution of the enzyme throughout the large membrane area of the endoplasmic reticulum, showed no detectable staining of a variety of acetone-fixed cells of non-bovine origin, including human epithelial (HeLa) cells, simian kidney (COS-1) cells, baby hamster kidney fibroblast cells, rat fibroblast cells, and murine L fibroblast cells. These results indicate that the affinity-purified antibodies are bovine-specific.

The affinity-purified antibodies specifically absorbed Gal T activity from 1% Triton X-100 extracts of MDBK cells; therefore, the antibodies purified from the SDS-solubilized fusion protein recognized the enzyme in its native conformation. On the other hand, the anti-bovine Gal T

\[ \text{Immunofluorescence of acetone-fixed MDBK cells with the affinity-purified antibodies gave a discrete juxtanuclear staining.} \]

\[ ^4 \text{C. Kooy, unpublished observations.} \]
Gal T in transfected cells, a cDNA containing the complete coding sequence of the enzyme was cloned into the pSVTgpt expression vector, as shown in Fig. 1b. The expression of bovine Gal T was initially examined in transient expression experiments using COS-1 cells, and the product was detected by indirect immunofluorescence with the affinity-purified antibodies to bovine Gal T. Staining of acetone-fixed transfected COS-1 cells revealed high levels of intracellular bovine Gal T, with a predominant juxtanuclear localization, indicative of the Golgi complex (Fig. 4a). A similar juxtanuclear staining pattern was observed with the fluorescent sphingolipid, C<sub>6</sub>-NBD-ceramide, in COS-1 cells (Fig. 4c). In some transfected cells, the product was also observed to be associated with a reticular network, probably the endoplasmic reticulum, and also with intracellular vesicles. This result probably reflects different levels of expression between transfected cells. Bovine Gal T could not be detected at the cell surface of transfected COS-1 cells by indirect immunofluorescence of nonpermeabilized cells (data not shown). As a control for cell surface expression, COS-1 cells were also transfected with a construct containing the cDNA for the human transferrin receptor (pSVT-TfR). As expected, abundant cell surface expression of the human transferrin receptor was detected in these transfected cells using the monoclonal antibody OKT9 specific for the human receptor (Fig. 4b).

pSVTgpt-GalT-transfected COS-1 cells showed a 25-fold increase in Gal T activity, as compared with untransfected COS-1 cells. Therefore, as the transfection efficiency was approximately 10%, the bovine Gal T-expressing COS-1 cells have an increase in Gal T activity of approximately 250-fold over endogenous levels. These results indicate that the mechanism for Golgi localization of bovine Gal T is conserved across species and that the Golgi retention of this enzyme is not readily saturated.

As transient expression in COS-1 cells produces very high levels of intracellular bovine Gal T, it was important to confirm the localization in an expression system where the antibodies only weakly immunosorbed the corresponding activity from the nonbovine cell extracts; 34 and 27 times more antibody was required to immunosorb 50% of Gal T activity from COS-1 or murine L cell extracts, respectively, compared with the bovine cell extract (Fig. 3d). Thus, the antibodies showed a marked specificity for the bovine Gal T, results that are consistent with the immunofluorescence data. Therefore, these antibodies provide a suitable reagent to study the Golgi localization of the bovine enzyme expressed in transfected mammalian cells.

Expression of the Full-length Bovine Gal T cDNA in Simian COS-1 Cells and Murine L Cells—For expression of bovine
levels resemble the endogenous level of Gal T. In addition, stably expressing cell lines were required to allow a more detailed investigation of the localization of the product. Therefore, murine L cell lines were generated that stably express bovine Gal T. The plasmid pSVTgpt-GalT was introduced into L cells, and transfectants were selected in medium containing mycophenolic acid, xanthine, hypoxanthine, amnoinoterin, and thymidine. Indirect immunofluorescence of permeabilized, transfected L cells with the affinity-purified anti-Gal T antibodies revealed strong perinuclear staining (Fig. 5a). As expected, the expression of bovine Gal T was heterogeneous in the uncloned population of L cells. Some cells showed no detectable reactivity with the antibody, again illustrating the lack of cross-reactivity of the antibodies with the endogenous murine Gal T. L cell clones were isolated by limiting dilution, and individual clones were screened by indirect immunofluorescence of acetone-fixed and permeabilized cells. The staining pattern of a typically high expressing clone (L cell clone 8) is shown in Fig. 5b. All the cells in the cloned population showed a similar staining pattern with the anti-Gal T antibodies; again, a distinct perinuclear staining pattern was clearly evident. Pretreatment of this L cell clone with nocodazole resulted in a vesicular staining pattern with the anti-Gal T antibodies, and treatment with brefeldin A resulted in a redistribution of the expressed product into an endoplasmic reticulum-like staining pattern (Fig. 5d and e), results that are consistent with a Golgi localization of the expressed product.

A number of investigators have reported that Gal T is localized not only to the Golgi but also at the cell surface of a variety of cells, including MDBK cells (Sherer et al., 1985). Therefore, we analyzed the high expressing L cell clone 8 for the presence of cell surface bovine Gal T, using the polypeptide-epitope-specific antibodies. Cell surface expression of bovine Gal T could not be detected in the high expressing clone by indirect immunofluorescence of nonpermeabilized cells (data not shown). However, analysis of the clones by the more sensitive technique of flow cytometry consistently detected low levels of cell surface bovine Gal T determinants (Fig. 5f). It is likely that this surface-reactive material is derived from the expression of transfected bovine Gal T cDNA as 1) untransfected L cells showed no surface reactivity with the affinity-purified antibodies (Fig. 5f) and 2) the cells were grown in serum-free medium for 16 h prior to analysis to eliminate possible contamination of bovine Gal T derived from the fetal calf serum in the medium. As expected, following permeabilization of the fixed L cell clone, a high level of intracellular bovine Gal T was detected by FACs analysis (data not shown). Comparison of the fluorescence intensity of the permeabilized and nonpermeabilized cells indicated that less than 10% of the total immunoreactivity was associated with the cell surface.

The transfected L cell clone 8 had a 3.5-fold increase in β1,4-Gal T activity, as compared with nontransfected L cells. Furthermore, lactose synthetase activity showed a similar increase in this transfected L cell clone, confirming the specificity of the Gal T cDNA. Immunoprecipitation of metabolically labeled transfected cell extracts with the affinity-purified antibodies identified a major component of 54 kDa and a minor component with a lower molecular mass of 46 kDa (Fig. 6A, lane 4). These components were not detected in untransfected L cells (Fig. 6A, lanes 1 and 2) or in transfected cells with preimmune immunoglobulin (Fig. 6A, lane 3). A background band of 43 kDa was detected in all immunoprecipitates. To investigate whether the difference in size between the 54- and 46-kDa components was due to N-glycosylation, the immunoprecipitated bovine Gal T from the L cell clone was digested with peptide N-glycosidase F. The 54-kDa component was found to be susceptible to peptide N-glycosidase F digestion, and after 16 h of digestion, was converted predominately to the 46-kDa component, with a minor component of 49 kDa also detected (Fig. 6B). The 46-kDa component is the expected size of the polypeptide of bovine Gal T (D’Agostaro et al., 1989). The higher molecular weight component of 49 kDa probably represents partially deglycosylated material. Overall, these results show that the majority of the bovine Gal T expressed in L cells carries N-glycans, whereas the minor 46-kDa species represents non-N-glycosylated bovine Gal T.

Expression and Localization of Deletion Mutants of Bovine Gal T—The role of the amino-terminal cytoplasmic domain of bovine Gal T in Golgi localization was investigated by the construction of a deletion mutant that lacked 18 amino acids of the 24-amino acid cytoplasmic tail (Fig. 1c). The remaining six amino acids include three positively charged residues that may be critical for correct membrane orientation (Beltz et al., 1991; Parks and Lamb, 1991). The construct introduced two additional amino acids at the amino terminus, namely, a translation initiation methionine and an alanine residue.
were immunoprecipitated with preimmune immunoglobulin with \([\text{RSS}]\)methionine and \([\text{SS}]\)cysteine, and the solubilized extracts polyacrylamide gel, and the resulting fluorograph is shown. The molecular mass standards are indicated. A major component of 51 kDa was immunoprecipitated from pSVTgpt-GalT-transfected COS-1 cells (Fig. 7A). The 48-kDa component from the pSHT-GalT(T,-S/A) transfection is approximately the size expected for the truncated bovine Gal T lacking the cytoplasmic tail and membrane anchor.

A pulse-chase experiment was performed to determine if the truncated bovine Gal T was secreted from the COS-1 cells. Cells transfected with pSHT-GalT(T,-S/A) were incubated for 30 min in medium containing \([\text{SS}]\)methionine and \([\text{SS}]\)cysteine and then chased in complete medium. At 1- and 4-h chase intervals, both the cell extracts and chase medium were incubated with affinity-purified Gal T antibodies, and the immunoprecipitates were examined by fluorography following SDS-PAGE. By 1 h of chase, the majority of the product was detected in the culture medium, and by 4 h of chase, all the product was found in the medium (Fig. 7A). Therefore, bovine Gal T lacking the cytoplasmic tail and membrane anchor is rapidly secreted from the transfected cells.

**Fig. 6. Immunoprecipitation of metabolically labeled bovine Gal T from transfected L cells.** A, untransfected L cells (UT) (lanes 1 and 2). L cells transfected with pSVTgpt-GalT (lanes 3 and 4), and pSVTgpt-GalT(−3/4T) (lanes 5 and 6) were labeled with \([\text{SS}]\)methionine and \([\text{SS}]\)cysteine, and the solubilized extracts were immunoprecipitated with preimmune immunoglobulin (lanes 1, 3, and 5) or affinity-purified anti-Gal T antibodies (lanes 2, 4, and 6), as described under "Experimental Procedures." B, peptide N-glycosidase F digestion of immunoprecipitated bovine Gal T proteins. Bovine Gal T was immunoprecipitated from radiolabeled cell extracts of pSVTgpt-GalT- and pSVTgpt-GalT(−3/4T)-transfected L cell clones, as above, and the isolated immune complexes were incubated for 16 h at 37 °C with peptide N-glycosidase F (N-glycanase) or buffer alone, as indicated. The digests were separated on a reducing SDS-polyacrylamide (10%) gel, and the resulting fluorograph is shown. The band indicated with an asterisk is a nonspecific component.

COS-1 cells were transfected with this pSVTgpt-GalT(−3/4T) construct; the Gal T product showed a juxtanuclear localization by immunofluorescence similar to that of the wild-type bovine Gal T (Fig. 4d). Stably expressing L cell clones were also generated with this deletion mutant, and the resulting cloned L cell populations showed a perinuclear staining pattern with the bovine anti-Gal T antibodies (Fig. 5c). FACS analysis of this clone also detected a low level of surface expression similar to that of the full-length Gal T (Fig. 5f). The bovine Gal T product was immunoprecipitated from metabolically labeled transfected L cell extracts with the affinity-purified antibodies, and the immunoprecipitates were analyzed by SDS-PAGE and fluorography. Two components were specifically immunoprecipitated, a major component of 51 kDa and a minor component of 44 kDa. Treatment of the immunoprecipitate with peptide N-glycosidase F resulted in the loss of the 51-kDa component with a corresponding increase in the 44-kDa component, as well as the appearance of an intermediate component of 48 kDa (Fig. 6B). These results are similar to the wild-type bovine Gal T, except that the deglycosylated product from the pSVTgpt-GalT(−3/4T)-transfected L cells is approximately 2 kDa smaller than the wild-type bovine Gal T, a size difference compatible with the deletion of 18 amino acids of the cytoplasmic tail. As the majority of the expressed protein contained N-glycans, the deletion mutant must be oriented correctly in the membrane in an N-\text{Asp}/C\text{out} fashion. The Gal T(−3/4T) appeared to be enzymatically active, as the transfected L cell clone displayed a 2.5-fold increase in Gal T activity, as compared with untransfected L cells.

The effect of deletion of both the cytoplasmic tail and the membrane anchor domains of bovine Gal T on intracellular localization was next investigated. As the membrane anchor of Gal T is a dual signal/anchor domain, the construct pSHT-GalT(T,-S/A) included a cleavable signal sequence, derived from influenza hemagglutinin, to allow the product to be translocated across the endoplasmic reticulum (Fig. 1d). The expressed protein in COS-1 cells transfected with pSHT-GalT(T,-S/A) was localized by indirect immunofluorescence of permeabilized cells using the affinity-purified anti-Gal T antibodies (Fig. 4e). The staining pattern was predominantly reticular, suggestive of the endoplasmic reticulum. Components of 48 and 45kDa were specifically immunoprecipitated from cell lysates of transfected COS-1 cells metabolically labeled for 30 min (Fig. 7A). The basis for the size difference between the two components is not known, but may be due to differences in the degree or nature of glycosylation. A major component of 51 kDa was immunoprecipitated from pSVTgpt-GalT-transfected COS-1 cells (Fig. 7A). The 48-kDa component from the pSHT-GalT(T,-S/A) transfection is approximately the size expected for the truncated bovine Gal T lacking the cytoplasmic tail and membrane anchor.

**Fig. 7. Secretion of soluble bovine Gal T from transfected COS-1 cells.** A, pulse-chase labeling of COS-1 cells transfected with the plasmids pSVTgpt-GalT and pSHT-GalT(T,-S/A). 48 h after transfection, the cells were pulse-labeled for 30 min with \([\text{SS}]\)methionine and \([\text{SS}]\)cysteine and then chased in complete medium. At 1- and 4-h chase intervals, both the cell extracts and chase medium were incubated with affinity-purified Gal T antibodies, and the immunoprecipitates were examined by fluorography following SDS-PAGE. By 1 h of chase, the majority of the product was detected in the culture medium, and by 4 h of chase, all the product was found in the medium (Fig. 7A). Therefore, bovine Gal T lacking the cytoplasmic tail and membrane anchor is rapidly secreted from the transfected cells.

The band indicated with an asterisk is a nonspecific component.
COS-1 cells with a half-time of <1 h (Fig. 7A). On the other hand, the product from pSVTgpt-GalT transfected COS-1 cells was found to be exclusively associated with the cell lysate at the completion of the chase period, and no product was detected in the culture medium (Fig. 7A). Based upon the intensity of the bands, a considerable proportion of the wild-type Gal T could not be accounted for after a 4-h chase. Although only semiquantitative, the result indicates considerable intracellular degradation of the full-length bovine Gal T in COS-1 cells during the 4-h chase period. It is of interest that the secreted product from pSHT-GalT(-T, -S/A)-transfected cells is a 53-kDa species, which is a higher molecular mass than the major intracellular forms of the truncated product, or the 51-kDa membrane-associated wild-type bovine Gal T. This increase in size could be due to terminal glycosylation events, for example sialylation, immediately prior to secretion. The molecular mass of the wild-type Gal T did not increase during the chase period, indicating that the bulk of the membrane Gal T was inaccessible to the further modification observed with the truncated secreted form.

To exclude the possibility that the stem domain is rapidly cleaved from the soluble form of bovine Gal T prior to secretion, a construct containing only the catalytic domain of Gal T was also prepared using the pSHT vector (Fig. 1e). Expression of pSHT-GalT(-T, -S/A, -Stem) in COS-1 cells resulted in secretion of the product at a rate similar to that of the pSHT-GalT(-T, -S/A) construct (data not shown). The secreted product of the pSHT-GalT(-T, -S/A, -Stem) transfection has an apparent molecular mass of 41 kDa by SDS-PAGE (Fig. 7B). As the stem domain contains one of the two potential N-glycosylation sites of bovine Gal T, the 12-kDa size difference between the two secreted Gal T products is consistent with a 61-amino acid stem domain together with an N-linked oligosaccharide. The size difference also indicates that the secreted product from the pSHT-GalT(-T, -S/A) transfected cells contains the majority of the stem region.

The Signal/Anchor Domain of Bovine Gal T Contains a Golgi Retention Signal—The results obtained with the deletion mutants of Gal T indicate that either bovine Gal T has to be membrane-anchored for the retention signals to be effective, or alternatively, the transmembrane domain itself contains the signals for Golgi localization. This question has been examined by the expression and localization of hybrid molecules in transfected COS cells. To assess whether the Gal T transmembrane domain is essential for Golgi localization, the signal/anchor domain of bovine Gal T was replaced with the signal/anchor domain of the human transferrin receptor (Fig. 1f). The transferrin receptor has an N<sub>c</sub>/C<sub>m</sub> orientation similar to that of Gal T but, unlike Gal T, is expressed predominantly at the cell surface (see Fig. 4b). COS cells were transfected with pSVTgpt-GalT/TfR(S/A) and indirect immunofluorescence of permeabilized cells with the anti-Gal T antibodies showed that the Gal T/TfR(S/A) hybrid molecule was not retained in the Golgi but was transported efficiently to the cell surface (Fig. 8a). pSVTgpt-GalT/TfR(S/A)-transfected COS cells showed about a 25-fold increase in Gal T activity, as compared with untransfected COS cells, indicating that the catalytic domain of the hybrid protein was corrected folded. The cell surface localization of the hybrid molecule indicates that the signal/anchor domain of Gal T is necessary for Golgi localization.

To determine if the Gal T signal/anchor domain is sufficient for Golgi retention, we have examined the ability of this hydrophobic domain to retain the secreted molecule, ovalbumin, within the Golgi. The hybrid construct contained a truncated cytoplasmic tail of the transferrin receptor, the bovine Gal T signal/anchor domain, and amino acids 139–385 of ovalbumin (Fig. 1g). The latter represents the carboxy-terminal domain of ovalbumin, which excludes the unusual internal signal sequence of this molecule (Meek et al., 1982). Ova/Gal T(S/A) hybrid molecules were localized in permeabilized transfected COS cells with anti-ovalbumin antibodies and found in cell surface fluorescence with constructs a and c but not with construct b (data not shown). C, carboxy terminus; N, amino terminus. Magnification ×270.

![Fig. 8. Cellular localization of hybrid proteins in transfected COS-1 cells by indirect immunofluorescence. COS-1 cells transfected with the constructs pSVTgpt-GalT/TfR(S/A) (a), pSVTgpt-Ova/GalT(S/A) (b), and pSVT-Ova/TfR (c) were fixed and permeabilized with acetone and stained by indirect immunofluorescence with the affinity-purified anti-Gal T antibodies (a) or anti-ovalbumin antibodies (b and c). Staining of nonpermeabilized transfected cells also resulted in cell surface fluorescence with constructs a and c but not with construct b (data not shown). C, carboxy terminus; N, amino terminus. Magnification ×270.](image-url)
expression of bovine Gal T in transfected murine L cells. Bovine Gal T was found to be efficiently localized to the Golgi complex in both transfected cells. Using these transfection systems we have explored the molecular basis for the sorting and retention of Gal T to the Golgi complex. From the intracellular localization of deletion mutants of bovine Gal T and of hybrid Gal T molecules, we conclude that the majority of the cytoplasmic tail is not required for Golgi localization, whereas the transmembrane domain of bovine Gal T contains a positive signal for the retention of the glycosyltransferase within the Golgi complex.

Bovine Gal T was localized in transfected cells with antibodies raised to the native, catalytic domain of bovine milk Gal T. As the antibodies were affinity-purified from a recombinant Gal T-fusion protein, they are specific for polypeptide epitopes. Previous studies have shown that polyclonal antibodies to human milk Gal T recognized blood group-related carbohydrate antigens present on the glycoprotein enzyme; therefore these antibodies are likely to cross-react with other glycocoagglutinins (Childs et al., 1986). The polypeptide-epitope-purified antiserum generated by the bovine clone therefore recognized the epitope. Although the recombinant fusion protein was insoluble in Triton X-100 and was coupled to Sepharose 4B solubilized in SDS, the antibodies purified from the denatured fusion protein nonetheless recognized native bovine Gal T, as they effectively immunoabsorbed the Gal T activity from cell extracts, as well as recognizing denatured bovine Gal T. Significantly, the antibodies showed minimum cross-reactivity with Gal T from non-bovine sources; therefore, the antibodies appear to recognize predominantly bovine-specific epitopes. This species specificity of the anti-Gal T antibodies is probably not surprising as Gal T is a common Golgi enzyme. Any newly synthesized antibodies capable of recognizing conserved luminal Gal T epitopes would be likely to interact with antigen during transport through the Golgi of the B cell and thereby be inhibited from secretion. On the other hand, antibodies recognizing species-specific luminal epitopes of Gal T would not be impeded during secretion.

Bovine Gal T was expressed at elevated levels in both COS-1 and L cells, as compared with the endogenous Gal T activity, and was localized predominantly to the Golgi complex. Indirect immunofluorescence did not detect surface expression of bovine Gal T in the stably expressing L cells. However, flow cytometry detected a low level of surface Gal T determinants in L cells expressing either the full-length Gal T or the -3/4 T deletion mutant. This surface immunoreactive material is probably derived from the expression of the transfected Gal T cDNA, as the cells were grown in serum-free medium to eliminate possible contamination by fertilally derived bovine Gal T. However, the precise nature of the product expressed at the cell surface is not known. There have been many reports of the expression of Gal T at the surface of a variety of cells based on immunohistochemical methods; these studies have been difficult to interpret as they have not employed antibodies specific for polypeptide epitopes. The results presented here, using polypeptide-epitope-purified antibodies, clearly show that bovine Gal T can be expressed on the cell surface of transfected cells. However, the biological relevance of the low level of surface expression in these cells is not clear and may simply reflect a low level of leakage from the Golgi complex. A recent study using a monoclonal antibody to the protein moiety of Gal T also reported the detection of cell surface Gal T in a variety of cultured cells and tissues by immuno-electron microscopy (Suganuma et al., 1991).

Bovine Gal T has two potential N-glycosylation sites, with one on the catalytic domain (amino acid residue 117) and one on the stem region (amino acid residue 90); amino acid sequencing of tryptic peptides of bovine Gal T suggests that both sites carry N-glycans (D'Agostaro et al., 1989). Digestion of the immunoprecipitated product from the transfected L cell lines with peptide N-glycosidase F showed that the majority of the full-length bovine Gal T and the -3/4 T deletion mutant were N-glycosylated, indicating that both proteins were correctly orientated within the Golgi membrane of these transfected cells, i.e. N_o/C_o orientation. For both constructs, a small amount of product was also detected that lacked N-glycans. This is probably due to heterogeneity of glycosylation, although the possibility of an N_o/C_o orientation (type I membrane protein) for these species cannot be eliminated. Deglycosylation of the immunoprecipitated bovine Gal T resulted in a predominant species of 46 kDa for the full-length Gal T and of 44 kDa for the -3/4 T deletion mutant. There are two in-frame AUG initiation codons in the long form of the bovine Gal T transcript (Russo et al., 1990) that are of approximately equal strength for translational initiation. Translation initiation from the first AUG codon would result in a full-length 45-kDa polypeptide, whereas initiation from the second AUG would result in a 43.5-kDa polypeptide lacking 13 amino acids of the 24-amino acid N-terminal cytoplasmic domain. As a 43.5-kDa species was not observed after deglycosylation of the product from the pSVTgpt-GaT transfected L cells, the first AUG initiation codon appears to dominate, in agreement with the linear scanning model of translational initiation (Kozak, 1986, 1989). Similar conclusions, using in vitro translation studies, have recently also been reported for bovine Gal T by Russo et al. (1990).

Gal T was effectively retained in the Golgi of COS-1 cells, even though these transfected cells contained greater than a 100-fold increase in Gal T activity. Therefore, the mechanism for Golgi localization is conserved across species and is not readily saturated. Deletion of 18 of the 24 amino acids in the cytoplasmic domain did not affect Golgi retention of the bovine Gal T in transfected COS-1 or L cells. It has been proposed that the two forms of Gal T, which differ in the length of the cytoplasmic domain, may have different intracellular localizations, i.e. trans-Golgi compared to cell surface (Russo et al., 1990; Lopez et al., 1991). However, removal of the bulk of the cytoplasmic domain did not result in an increase in the level of cell surface expression of bovine Gal T in transfected L cells. Furthermore, the result clearly indicates that the majority of the cytoplasmic domain is not required for retention within the Golgi complex. Although the immunofluorescence data, together with drug treatments, is consistent with a Golgi localization, precise localization of the full-length bovine Gal T and the deletion mutant within the Golgi stack of the transfected cells will require ultrastructural localization. The stably expressing L cells clones will therefore be extremely valuable for these immuno-electron microscopy studies.

In contrast to the full-length Gal T and the -3/4 T deletion mutant, truncated bovine Gal T lacking the transmembrane domain was rapidly secreted from COS-1 cells and was not retained within the Golgi complex. The transmembrane domain of Gal T was shown to be essential for Golgi localization, as replacement of the transmembrane domain of Gal T with the transmembrane domain of the transferrin receptor resulted in efficient transport of the hybrid molecule to the cell surface. Furthermore, the transmembrane domain of Gal T efficiently retained the secreted protein ovalbumin to the Golgi complex; therefore, this domain clearly contains a positive retention signal for the localization of proteins to the Golgi. Whether this retention signal is sufficient for the
localization to the trans compartment of the Golgi is not yet known and will require the localization of the ovalbumin hybrid molecules at the electron microscope level.

Although cDNAs have been isolated for a number of Golgi glycosyltransferases, comparison of these primary amino acid sequences has not revealed an obvious linear Golgi retention signal in the transmembrane domains of these proteins. Ben-diaik (1990) identified a possible peptide motif of Ser(Thr)-X-Glu(Gln)-Arg(Lys) found in close proximity to the hydrophobic signal/anchor domain of a number of glycosyltransferases. This motif is found on the cytoplasmic domain of bovine Gal T and, furthermore, as it was disrupted in the Gal T (−3/4T) deletion mutant, is unlikely to be important in Golgi localization.

The results of the Gal T deletion mutants are in contrast to late-Golgi localized a2,6-sialyltransferase, where the soluble form of the enzyme containing the stem region was found to be retained in the Golgi and only very slowly secreted from transfected cells (Colley et al., 1989b); on the other hand, the secreted form of Gal T was found to be further retained in the Golgi and only very slowly secreted from transfected cells (Colley et al., 1989a). It is possible that the retention mechanism may differ between Gal T and a2,6-sialyltransferase. Although both enzymes are at the late-Golgi stage, a2,6-sialyltransferase and Gal T may reside in different Golgi compartments. In contrast to the trans-Golgi localization of Gal T, a2,6-sialyltransferase has been reported to be localized in the trans-Golgi network (Roth et al., 1985b). However, as double labeling studies using defined antibodies to both glycosyltransferases have not yet been performed, this issue remains unresolved. It is of particular interest, then, that the secreted form of Gal T was found to be further modified post-translationally prior to secretion, which is a modification not observed for the Golgi-retained form of Gal T. The possibility that this modification involves the addition of sialic acid residues in a compartment later than the membrane-bound Gal T Golgi compartment is currently being explored.

Significantly, the El glycoprotein of an avian coronavirus, which is targeted to the cis-Golgi, has been shown to require the first membrane-spanning domain to be retained in the Golgi (Machamer and Rose, 1987; Machamer et al., 1990). Furthermore, this membrane domain of E1 has recently been shown to effectively retain different plasma membrane proteins in the Golgi of transfected cells (Swift and Machamer, 1991). Site-directed mutagenesis has indicated that uncharged polar residues within the membrane anchor are an important feature of the Golgi retention signal of this viral glycoprotein (Swift and Machamer, 1991). As we have shown here that the transmembrane domain of the endogenous Gal T also contains the retention signal for Golgi localization, it is possible that the sequences associated with transmembrane domains will be a general feature of Golgi retention signals.

The retention signals for the membrane proteins resident in the endoplasmic reticulum are found within the hydrophilic cytoplasmic domain (Jackson et al., 1990). Thus, the identification of hydrophobic retention signals for Golgi proteins suggests a novel mechanism for the localization of intracellular proteins. As the retention of Gal T in transfected COS cells was not readily saturated, it would appear unlikely that the mechanism of retention involves a specific receptor-based system. An alternative mechanism may involve aggregation and/or an interaction between membrane lipids and the signal/anchor domain. Elucidation of the precise nature of the Gal T retention signal and the mechanism of Golgi retention is fundamental to an understanding of this complex organelle.

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