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Expression of bovine β-1,4-galactosyltransferase cDNA in COS-7 cells

(genomic clone/expression vector/monoclonal and polyclonal antibodies)

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ABSTRACT **A bovine β-1,4-galactosyltransferase (GT; EC 2.4.1.90) cDNA in an Okayama–Berg vector, pLsGT, was constructed from a partial cDNA clone and a genomic fragment. We report that the cDNA sequence of pLsGT, in a transient expression assay in COS-7 cells, codes for an enzymatically active GT protein. There is an approximately 12-fold increase in the GT activity in pLsGT-transfected cells compared to cells transfected with the antisense bovine GT construct, pLasGT, or pSV_2Neo or mock-transfected cells. The increased activity is correlated with the increase in bovine GT mRNA, which is distinguishable from COS GT mRNA with a 3′-end-specific probe of pLsGT. The expressed GT activity is modulated by α-lactalbumin, which changes the acceptor specificity to glucose to synthesize lactose. Polyclonal antibody raised against SDS/PAGE-purified bovine milk GT and a monoclonal antibody (mAb 4-10) directed against a synthetic peptide corresponding to the amino-terminal region of the protein encoded by pLsGT bind the expressed protein, and the resulting immunoprecipitates exhibit GT enzymatic activity.**

N-Acetylglucosamine β-1,4-galactosyltransferase (GT; EC 2.4.1.90) is but one member of the glycosyltransferase family of enzymes. Many of these enzymes are involved in the synthesis and extension of oligosaccharide chains on glycoproteins and glycolipids, generating a series of disaccharide linkages (1–3). Molecular approaches have been utilized to study the various transferases and cDNAs for some transferases have been cloned (4–9).

GT transfers galactose from UDP-galactose to *N*-acetylglucosamine (GlcNAc) to produce *N*-acetyllactosamine with a β-1,4-glycosidic linkage. The cDNA for GT has been independently cloned by several laboratories from lactating bovine mammary gland (4), the bovine kidney cell line MDBK (5), human liver (6), F9 embryonal carcinoma cells (7), and the mouse mammary cell line c127 (8). All these clones show strong homology to each other. On the other hand the 5′ sequence of human liver GT cDNA clone isolated by Humphreys-Beher *et al.* (10) does not exhibit homology to any of the reported GT cDNAs (4–8), in spite of the fact that the deduced partial amino acid sequence of the cDNA clone showed partial homology to the amino-terminal sequence of the bovine GT protein they isolated. This raises the question whether the described clones (4–8) do indeed code for active GT.

To address this problem, we constructed a full-length bovine GT cDNA in an Okayama–Berg expression vector (pLsGT)*, transfected it into COS-7 cells, and obtained a 12-fold increase in GT enzymatic activity in the transfected COS-7 cells compared to mock-transfected cells. This GT activity is modulated by α-lactalbumin, which changes the acceptor specificity from GlcNAc to glucose. In addition, this GT activity is immunoprecipitable by a polyclonal antibody raised against SDS/PAGE-purified bovine milk GT and by a monoclonal antibody (mAb) directed against a synthetic peptide whose sequence was derived from the cDNA sequence that spans a portion of the amino-terminal region of GT. In contrast to pLsGT, pLasGT, another construct that has a region of the 5′ end sequence of bovine GT cDNA in the reverse orientation, does not show any increase in the GT activity compared to the mock-transfected cells.

MATERIALS AND METHODS

Enzymes and Chemicals. All restriction enzymes, T4 DNA ligase, and reverse transcriptase were purchased from New England Biolabs, Bethesda Research Laboratories, International Biotechnologies, or Boehringer Mannheim and were used according to the manufacturer's directions. Bovine milk GT was purchased from Sigma and further purified. [^{32}P]-dCTP (3000 Ci/mmol; 1 Ci = 37 GBq) and UDP-[^{3}H]galactose (20 Ci/mmol) were purchased from Amersham.

Cell Cultures. Stock cultures of monkey kidney COS-7 cells were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal calf serum (GIBCO), L-glutamine (2 mM), penicillin (100 units/ml), streptomycin (100 μg/ml), and gentamycin (500 μg/ml).

General DNA Recombinant Techniques and DNA Sequence Analysis. Plasmid preparations, restriction endonuclease digestions, ligations, kinase reactions, phosphatase treatment, and end-labeling of DNA fragments were carried out as described (11). Nucleotide sequence analysis was carried out by the Maxam–Gilbert method (12) and by the chain-termination method (13) using subclones of the cDNA restriction fragments in phage M13 vectors (14).

Screening of a Genomic Library for the 5′ End Sequence of the Bovine GT Gene and Plasmid Construction. The genomic library made from bovine placenta constructed in Charon 28 λ phage (15) was kindly provided by F. M. Rottman (Case Western Reserve, Cleveland). A synthetic 100-mer oligonucleotide corresponding to the extreme 5′ end sequence of pLbGT-52-1, the longest cDNA clone, and the *Sst* I–*Kpn* I fragment of pLbGT-1 (4) were used as hybridization probes to screen the library. The 100-mer oligonucleotide was synthesized on Pharmacia's Gene Assembler using the phosphoramidite procedure and purified on Pharmacia's FPLC system using Pro-RPC and Mono-Q columns according to the protocols from Pharmacia. Hybridization with the 100-mer was carried out as described (4) except for hybridization and washing steps that were done at 50°C. Clone λbGT-9, which hybridized to the 100-mer and to the *Sst* I–*Kpn* I probes, was plaque purified. A 2.3-kilobase (kb) *Hin*dIII–*Hin*dIII fragment of λbGT9 DNA contained an *Xho* II–*Sst* I fragment (see

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Abbreviations: GT, β-1,4-galactosyltransferase; mAb, monoclonal antibody.

*The sequence reported in this paper has been deposited in the GenBank data base (accession no. M25398).

Results) to which only the 100-mer hybridized. This fragment was isolated, filled-in by reverse transcriptase, and blunt-end ligated with an *Sst* I-linker, CGAGCTCG (Pharmacia). The ligated sample was digested with *Sst* I, separated on a NAP-10 column (Pharmacia), and ligated to *Sst* I-digested and phosphatase-treated pLbGT-1 (4). The resulting plasmid construct was used to transform DH5α competent cells (BRL) and the transformants were screened with ^{32}P-labeled 100-mer oligonucleotide. The orientation of the insert in hybridization-positive clones was established.

Calcium Phosphate-Mediated Transfection of COS-7 Cells. Since the quality of plasmid DNA used for transfection is critical, the protocol of Chen and Okayama (16) was used to purify the plasmid DNA; this protocol includes two cycles of cesium chloride density gradient centrifugation of the plasmid DNA, a phenol/chloroform extraction, and two ethanol precipitations. The DNA was stored in 10 mM Tris·HCl, pH 7.5/1 mM EDTA (TE buffer). COS-7 cells were seeded at 5 $\times$ 10^5 cells per 100-mm dishes and, after an overnight incubation at 37°C in a 5% CO_2/95% air atmosphere, were transfected with 15 μg of DNA (1 μg/μl) using the calcium phosphate transfection protocol of Chen and Okayama (16). After 48 hr, the cells were washed twice with ice-cold isotonic phosphate-buffered saline (PBS), harvested by scraping the cells from each plate in 5 ml of PBS, and centrifuged at 7000 $\times$ *g* for 10 min. The cell pellet was resuspended in PBS, 3 $\times$ 10^6 cells were distributed per Eppendorf tube and centrifuged, and the pellet was stored at −70°C.

Preparation of Cell Extracts. Cell pellet in an Eppendorf tube containing 3.0 $\times$ 10^6 cells was resuspended in 200 μl of 20 mM Tris·HCl, pH 8.0/1% Triton X-100. The suspension was kept on ice for 20 min and gently homogenized using a pellet pestle (Kontes). The suspension was centrifuged at 7000 $\times$ *g* at 4°C for 15 min, and the supernatant was assayed. The protein concentration of the extracts from transfected cells, determined by the Bradford assay (11), ranged between 0.23 and 0.25 μg/μl of extract. One microliter of cell extract represented 1.5 $\times$ 10^4 cells in all transfection assays.

Measurement of GT Activity. Enzymatic assays were performed by a radiochemical procedure (17–19). Cell extracts were assayed at 30°C for 30 min in a 100-μl incubation mixture containing 2 μCi of UDP-[^{3}H]galactose (20 Ci/mmol), 5 nM UDP-galactose, 400 nM $MnCl_2$, and 1 μl of Triton X-100. In the GT activity assay, the incubation mixture contained 2 μM GlcNAc as an acceptor molecule. In the lactose synthetase assay, the 100-μl incubation mixture contained 8 μg of bovine α-lactalbumin (Sephadex G-100 column-purified) and 2 μM glucose as an acceptor molecule. Assays were performed with and without acceptor molecules to assess the extent of hydrolysis by the nucleotide pyrophosphatases. The reaction was terminated by adding 100 μl of ice-cold H_2O and the unincorporated UDP-[^{3}H]galactose was separated from ^{3}H-labeled products as described (19) on an anion-exchange column (AG 1-X8, Bio-Rad).

RNA Blot Analysis. Total cytoplasmic RNA isolated by the guanidinium isothiocyanate method (20) was subjected to Northern blot analysis (11). Blotting of the nucleic acid to Hybond-C extra (Amersham) and hybridization were performed according to the manufacturer's protocol. Probes were labeled to a specific activity of 10^9 cpm/μg of DNA using Amersham's random-primer labeling kit.

Preparation of Polyclonal and Monoclonal Antibodies. Commercially available bovine GT (UDPgalactose:D-glucose 4-β-D-galactosyltransferase, EC 2.4.1.22; Sigma) was further purified by using an α-lactalbumin-Sepharose column (21) and by preparative SDS/PAGE (22). A portion of the outside edges of the Coomassie blue-stained lane showed two bands corresponding to the 54-kDa and 43-kDa proteins. The gel strips corresponding to the two bands were excised from the unstained gel, lyophilized, pulverized, resuspended in PBS, and used for immunization. Blood was drawn from rabbits prior to immunization. The serum was titered for immunoreactivity against bovine milk GT by using an ELISA (23). Polyclonal antibody prepared against the 54-kDa band was used in the present studies.

Mouse mAbs were raised at the Frederick Cancer Research Facility by a standard method (24) against the synthetic peptide Asn-Ser-Ser-Lys-Pro-Arg-Ser-Arg-Ala-Pro-Ser-Asn-Leu-Asp-Ala, corresponding to residues 90–104 of the protein sequence encoded by a full-length bovine GT cDNA.

GT Immunoprecipitation and Measurement of the Enzymatic Activity in the Immunoprecipitates. Antigen–antibody reactions were carried out at 4°C overnight in a 200-μl incubation mixture containing 4 μl of cell extracts from either pLsGT- or pLasGT-transfected COS-7 cells, with various amounts of preimmune or polyclonal antisera or mAb culture supernatants, in 1$\times$ antigen–antibody buffer (10 mM Tris·HCl, pH 8.0/150 mM NaCl/10 mM GlcNAc). Samples were further incubated for 10–12 hr with 10 μl of goat anti-rabbit or anti-mouse IgG (Cappel Laboratories) as the second antibody. Samples were centrifuged at 25,000 $\times$ *g* at 4°C for 15 min. The supernatant was removed, the pellet was resuspended in 200 μl of 1$\times$ antigen-antibody buffer and assayed for GT activity at 30°C for 30 min after the addition of a 50-μl mixture containing 3 μCi of UDP-[^{3}H]galactose (20 Ci/mmol), 12.5 nM UDP-galactose, and 1 μM $MnCl_2$. The products were separated as described above.

RESULTS AND DISCUSSION

Screening of the Genomic Library for the 5′ End Sequences. pLbGT-1, a partial bovine GT cDNA clone (4), shown in Fig. 1, was isolated from a bovine mammary gland cDNA library constructed in an Okayama–Berg expression vector. This clone lacked the 5′ nucleotide sequence coding for the extreme amino-terminal region of the protein. Rescreening of this library gave pLbGT-52-1 (Fig. 1), the longest cDNA clone, that contained an additional 165 base pairs (bp) at the 5′ end compared to pLbGT-1 but that still lacked the translation start codon. To obtain the rest of the 5′ end sequences including the start codon we screened a genomic library. To ensure that the clones isolated contained sequences covering the 5′ region of the GT gene, the genomic library was screened with the 0.9-kb *Sst* I–*Kpn* I fragment of pLbGT-1 (Fig. 1) and with a synthetic 100-mer oligonucleotide that covered the extreme 5′ region of the longest cDNA clone pLbGT-52-1 (Fig. 1). Clone λbGT9 was isolated by this procedure. *Hin*dIII digestion of λbGT9 DNA produced six fragments, a 2.3-kb fragment of which hybridized to the *Sst* I–*Kpn* I fragment and the 100-mer probe. This 2.3-kb fragment was isolated and subcloned into Bluescript (Stratagene) and pLλ9H2.3 (Fig. 1), the resulting construct, was subjected to further restriction digestion analysis. A 650-bp *Sst* I–*Sst* I subfragment of the 2.3-kb *Hin*dIII insert of pLλ9H2.3 hybridized only to the 100-mer probe. Since the 100-mer synthetic oligonucleotide probe represented sequences upstream of the *Sst* I site of the cDNA clone pLbGT-1, we suspected that the 650-bp *Sst* I–*Sst* I subfragment from the 2.3-kb *Hin*dIII fragment represented the extreme 5′ end region of the bovine GT gene. To verify this, the 650-bp subfragment was isolated and subcloned into M13mp19 and the resulting clone, Q1, was sequenced by the Maxam–Gilbert method utilizing the internal *Xho* II site of the insert as well as the *Eco*RI and *Bam*HI polylinker sites of M13 surrounding the insert.

DNA sequence analysis of the *Sst* I–*Sst* I fragment showed that the nucleotide sequence at the 3′ region matched the 5′ end 165-bp sequence of pLbGT-52-1, the longest cDNA clone (nucleotides 477–642), which included the sequence of the

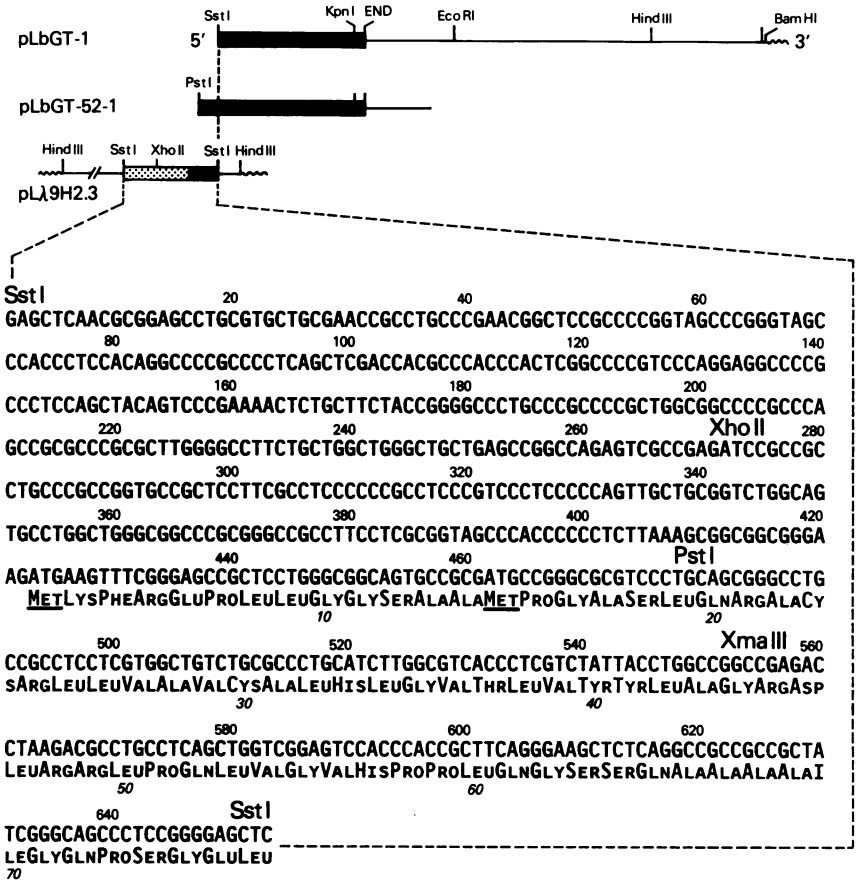

FIG. 1. Partial bovine GT cDNAs pLbGT-1, pLbGT-52-1, and pLλ9H2.3 (the genomic *Sst* I–*Sst* I fragment). The sequence of the *Sst* I–*Sst* I insert of pLλ9H2.3 corresponds to the 5′ end sequence of the bovine GT cDNA and the 5′ end flanking gene sequence. Solid boxes, coding sequences; stippled box, genomic sequence; squiggly lines, vector sequences.

100-mer synthetic oligonucleotide (nucleotides 477–576; Fig. 1). Furthermore, this sequence data showed that the 18 amino-terminal-end residues of the bovine GT are encoded by nucleotides 423–476. This cDNA-derived protein sequence includes the two in-frame methionines and is 72% homologous (13 of 18 residues) to the cDNA-derived amino-terminal-end sequence reported for murine GT (7, 8). Therefore, this *Sst* I–*Sst* I genomic DNA fragment contained the extreme 5′ coding region for bovine GT as well as 5′ non-coding cDNA sequences.

Construction of a GT Expression Vector. Nucleotides 1–422 of the *Sst* I–*Sst* I fragment (Fig. 1), preceding the first AUG codon, show no obvious eukaryotic promoter element ("TATA" box) or "CAAT" box (25). To ensure that the expression of the bovine GT cDNA was under the control of the simian virus 40 promoter of the Okayama–Berg vector, we used the *Xho* II–*Sst* I sequence (nucleotides 270–653) in the *Sst* I–*Sst* I fragment for construction of a cDNA clone containing the complete coding sequence of bovine GT (Fig. 2). The *Xho* II–*Sst* I fragment was filled-in, ligated to *Sst* I linker, and inserted into the *Sst* I site of pLbGT-1. The orientation of the insert in the resulting constructs was determined by using the unique *Xma* III and *Sal* I sites in the insert and vector, respectively. pLsGT contains the *Xho* II–*Sst* I insert in the sense orientation. This construct would code for a 401-amino acid protein with two in-frame methionine residues at the amino-terminal end. pLasGT has the *Xho* II–*Sst* I insert in the opposite orientation and would not code for a protein with any resemblance to GT.

Transfection of COS-7 Cells and Distinction of COS Cell vs. Bovine GT mRNA. COS-7 cells were transfected with pLsGT, pLasGT, or pSV_2Neo DNA or were mock-transfected. Since the 3′ non-coding region of GT mRNA is much less homologous among different species (unpublished results), we used the 3′ end non-coding *Hin*dIII–*Bam*HI fragment of pLsGT (Fig. 3) as a hybridization probe to distinguish endogenous COS-7 cell GT mRNA from bovine GT mRNA. The coding sequence for GT is highly conserved among different species. As shown in Fig. 3, total RNA isolated from mock-

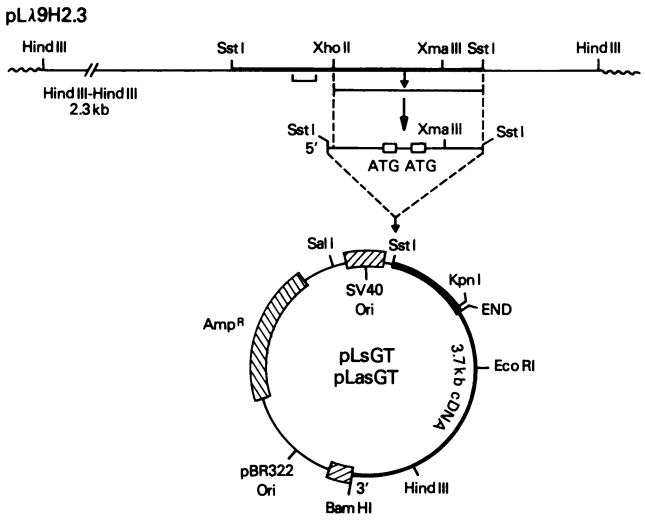

FIG. 2. Bovine GT constructs in an Okayama–Berg expression vector. An *Xho* II–*Sst* I genomic fragment within the *Sst* I–*Sst* I region of pLλ9H2.3 was ligated into the *Sst* I site of pLbGT-1 in sense (pLsGT) and antisense (pLasGT) orientations. Amp^r, ampicillin resistance; Ori, origin; SV40, simian virus 40.

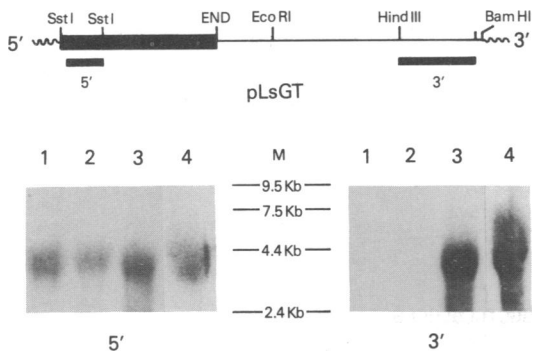

FIG. 3. Detection of bovine vs. COS-7 GT mRNA. (*Upper*) Location of the 5′ and 3′ end probes from pLsGT. (*Lower*) Total cytoplasmic RNA from mock-, pSV_2Neo-, pLsGT-, or pLasGT-transfected COS-7 cells (lanes 1–4, respectively; 20 μg per lane) were analyzed by Northern blot analysis using either the 5′ end *Sst* I–*Sst* I fragment (*Lower left*) or the 3′ end *Hin*dIII–*Bam*HI fragment (*Lower right*) of pLsGT as hybridization probes. RNA ladders were used as molecular size markers (M).

transfected or transfected COS-7 cells, probed with the *Sst* I–*Sst* I fragment of pLsGT (5′ probe), detected COS-7 and bovine GT mRNAs. In contrast, when the *Hin*dIII–*Bam*HI fragment of pLsGT (3′ probe) was used as a hybridization probe, only the bovine GT transcript was detected in pLsGT- and pLasGT-transfected COS cells (Fig. 3).

GT Activity and Its Modulation by α-Lactalbumin. Although COS-7 cells do show endogenous GT activity, there was an approximately 12-fold increase in the activity of extracts assayed in the presence of GlcNAc as an acceptor substrate from pLsGT-transfected cells compared to the extracts from pLasGT-, pSV_2Neo-, or mock-transfected cells (Fig. 4*A*). Activity of cell extracts from pLsGT-transfected cells was stimulated about 60-fold when assayed in the presence of GlcNAc compared with samples assayed without GlcNAc, showing that the activity was absolutely dependent on the added exogenous acceptor substrate. Conversely, the activity of the cell extracts from mock- or pLasGT-transfected cells was stimulated only 3- to 5-fold in the presence of GlcNAc. All these activities are dependent on the concentration of the cell extract. The increase in GT activity in pLsGT-transfected COS-7 cells is consistent with the increase in GT mRNA levels (Fig. 3 *Lower right*, lane 3). Although there was an increase in the mRNA levels in pLasGT-transfected COS-7 cells (Fig. 3 *Lower right*, lane 4), there was no increase in GT activity, indicating that this mRNA was not translated to produce a functional protein.

To rule out the possibility that our cDNA clone, instead of coding for bovine GT, codes for a protein that somehow indirectly influences endogenous COS-7 cell GT activity either by activating GT directly or by interacting with a GT inhibitor, the following mixing experiment was performed. Various amounts of cell extracts from mock- and pLsGT-transfected cells were mixed and assayed, and the activities were compared to the samples assayed individually. The result showed that the mixed activity was not greater than the sum of the individual activities (data not shown). This is consistent with the fact that the pLsGT cDNA codes for GT rather than an activator protein.

Upon interaction of α-lactalbumin with GT to form lactose synthetase, the monosaccharide binding property of the enzyme is altered so that glucose, normally a poor acceptor, becomes a good acceptor substrate (26). The expressed GT protein in pLsGT-transfected COS-7 cells can be modulated by bovine α-lactalbumin to accept glucose as a substrate to produce lactose (Fig. 4*B*). There is a 10-fold increase in the lactose synthetase activity in the extracts from pLsGT-transfected cells compared to the extracts from pLasGT- or mock-transfected cells, which is consistent with the observed increase in GT activity (Fig. 4*A*). These results show that the bovine GT protein expressed transiently in COS-7 cells upon pLsGT transfection is fully functional and that the binding sites for UDP-galactose, GlcNAc, glucose, and α-lactalbumin are intact and operative.

Immunological Identification of Bovine GT in Transfected COS-7 Cells. The enzyme synthesized in the pLsGT-transfected COS-7 cells reacted immunologically with the polyclonal antibody raised in rabbits against SDS/PAGE-purified bovine milk GT (by Western blot analysis, data not shown). Immunoprecipitates from extracts of pLsGT-transfected COS-7 cells, obtained by the precipitation of the antigen–antibody complex with the second antibody (goat anti-rabbit IgG), showed GT enzymatic activity

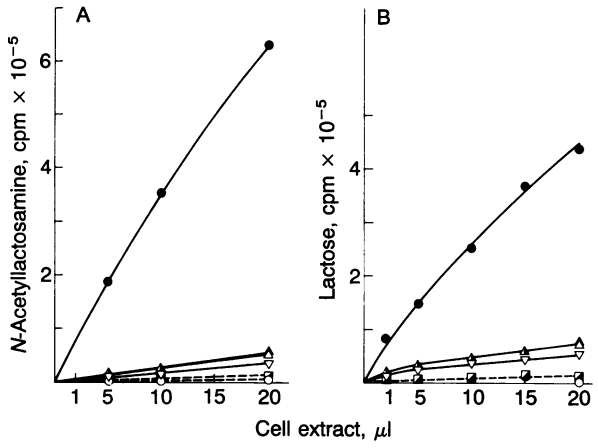

FIG. 4. Enzymatic activity of the extracts from transfected COS-7 cells. (*A*) GT activity of the cell extracts in the presence (—) or absence (---) of GlcNAc, the exogenous acceptor. The cpm from the sample containing no extract (3560 cpm) was subtracted from each data point. (*B*) Lactose synthetase activity of the extracts in the presence of 8 μg of bovine α-lactalbumin in the presence (—) or absence (---) of glucose. The cpm from the sample containing no cell extract (3200 cpm) was subtracted from each data point. The cpm for 20 μl of cell extract in −GlcNAc and −Glu samples ranged between 8000 and 15,000 and between 8000 and 10,000, respectively. ▲ and ■, mock; ▽ and ○, pSV_2Neo; △ and □, pLasGT; ●, pLsGT in the presence of GlcNAc or glucose; □, pLsGT in the absence of GlcNAc or glucose (the values are the same as for pLasGT and so the same symbol was used for both).

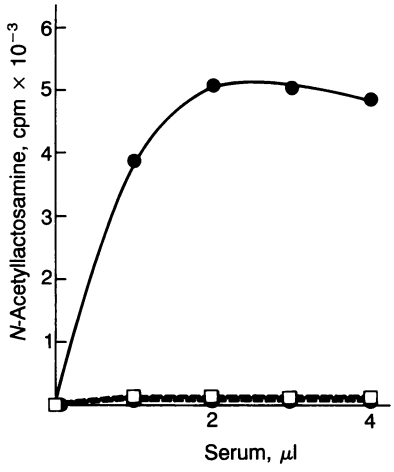

FIG. 5. GT activity of the immunoprecipitates using preimmune (dashed lines) and polyclonal (solid lines) antisera against bovine GT from cell extracts (4 μl) of pLsGT- (●) and pLasGT- (□) transfected cells. The cpm from samples containing 4 μl of extract with no serum (5650 cpm) was subtracted from each data point.

Table 1. GT activity of the immunoprecipitates using mAb 4-10

pLsGT cell extract, μl	mAb 4-10, μl	*N*-Acetyllactosamine, cpm × 10^{-3}
4	0	4,744
4	50	7,530
4	100	11,336
4	150	11,644
0	150	4,700

(Fig. 5). In contrast no enzymatic activity was detected in the immunoprecipitates from extracts of pLasGT-transfected cells. Since the assay conditions used to measure activity in the immunoprecipitates were not as described in Fig. 4*A*, the absolute values cannot be compared. The immunoprecipitate with the highest activity from the pLsGT sample, however, represents about 40% of the total GT activity of the sample measured under these conditions. There was no activity detected in immunoprecipitates obtained with preimmune serum.

A synthetic peptide that corresponds to the amino-terminal region of the protein encoded by the pLsGT cDNA was used to generate mouse mAbs. The peptide sequence is located 12 residues to the carboxyl-terminal side of the last leucine residue in Fig. 1 (4). These mAbs were tested for the highest antibody reactivity by Western blot analysis using pure bovine milk GT as the antigen (data not shown); hybridoma 4-10 gave the highest antibody titer. In immunoprecipitation experiments (Table 1), increasing amounts of mAb from hybridoma 4-10, precipitated increasing amounts of GT activity from pLsGT-transfected cell extracts. The samples with no mAb added or with mAb and no cell extracts showed background activity.

To summarize, we have shown that the full-length cDNA construct for bovine GT described here codes for an enzymatically active protein. The functional significance of the clone by Humphreys-Beher *et al.* (10) remains to be demonstrated. The constructs and the antibodies reported here, plus the α-lactalbumin constructs prepared earlier in this laboratory (27), should facilitate the delineation of the functional and interacting domains of GT and α-lactalbumin.

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