The Chicken Genome Contains Two Functional Nonallelic β1,4-Galactosyltransferase Genes

CHROMOSOMAL ASSIGNMENT TO SYNTENIC REGIONS TRACKS FATE OF THE TWO GENE LINEAGES IN THE HUMAN GENOME*

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Two distinct but related groups of cDNA clones, CKβ4GT-I and CKβ4GT-II, have been isolated by screening a chicken hepatoma cDNA library with a bovine β1,4-galactosyltransferase (β4GT) cDNA clone. CKβ4GT-I is predicted to encode a type II transmembrane glycoprotein of 41 kDa with one consensus site for N-linked glycosylation. CKβ4GT-II is predicted to encode a type II transmembrane glycoprotein of 43 kDa with five potential N-linked glycosylation sites. At the amino acid level, the coding regions of CKβ4GT-I and CKβ4GT-II are 52% identical to each other and 62% and 49% identical, respectively, to bovine β4GT. Despite this divergence in amino acid sequence, high levels of expression of each cDNA in Trichoplusia ni insect cells demonstrate that both CKβ4GT-I and CKβ4GT-II encode an α-lactalbumin-responsive, UDP-galactose-N-acetylgalcosamine β4-galactosyltransferase.

An analysis of CKβ4GT-I and CKβ4GT-II genomic clones established that the intron positions within the coding region are conserved when compared with each other, and these positions are identical to the mouse and human β4GT genes. Thus CKβ4GT-I and CKβ4GT-II are the result of the duplication of an ancestral gene and subsequent divergence. CKβ4GT-I maps to chicken chromosome Z in a region of conserved synteny with the centromeric region of mouse chromosome 4 and human chromosome 9p, where β4-galactosyltransferase (EC 2.4.1.38) had previously been mapped. Consequently, during the evolution of mammals, it is the CKβ4GT-I gene lineage that has been recruited for the biosynthesis of lactose. CKβ4GT-II maps to a region of chicken chromosome 8 that exhibits conserved synteny with human chromosome 1p. An inspection of the current human gene map of expressed sequence tags reveals that there is a gene noted to be highly similar to β4GT located in this syntenic region on human chromosome 1p. Because both the CKβ4GT-I and CKβ4GT-II gene lineages are detectable in mammals, duplication of the ancestral β4-galactosyltransferase gene occurred over 250 million years ago in an ancestral species common to both mammals and birds.

β1,4-Galactosyltransferase (β4GT; EC 2.4.1.38)1 is a constitutively expressed, trans-Golgi-resident, type II membrane-bound glycoprotein that catalyzes the transfer of galactose to N-acetylgalcosamine residues, forming the β4-N-acetyllactosamine (Galβ4-GlcNAc) or poly-β4-N-acetyllactosamine structures found in glycoconjugates (1). In mammals, β4GT has been recruited for a second biosynthetic function, the tissue-specific production of lactose, which takes place only in the lactating mammary gland. The synthesis of lactose is carried out by the protein heterodimer assembled from β4GT and the noncatalytic protein α-lactalbumin, a mammalian protein expressed exclusively in lactating mammary epithelial cells (2–4). Interestingly, β4GT from nonmammalian species such as chicken (4) and plant (5) can also functionally interact with α-lactalbumin in vitro, indicating that the α-lactalbumin binding domain in β4GT predates the rise of mammals.

We have reported that the murine β4GT gene is unusual in that it specifies two size sets of mRNAs in somatic cells of −3.9 and −4.1 kb. These two transcripts arise as a consequence of initiation at two different sets of start sites that are separated in the first exon by −200 bp. Because the respective start sites are positioned either upstream of the first of two in-frame ATGs (4.1 kb) or between these two in-frame ATGs (3.9 kb), translation of each mRNA results in the synthesis of two structurally related, trans-Golgi-resident protein isoforms that differ only in the length of their NH2-terminal cytoplasmic domain (6). The identical structural features are also found in the bovine (7) and human β4GT gene (8, 9), suggesting that they

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1The abbreviations used are: β4GT, β1,4-galactosyltransferase (EC 2.4.1.38); murine and human β4GT refer to the α-lactalbumin responsive, UDP-galactose:N-acetylgalcosamine β4-galactosyltransferase that has been mapped to the centromeric region of mouse chromosome 4 and human chromosome 9p13, respectively; bovine β4GT is the corresponding bovine homologue of the mouse and human galactosyltransferase; bp, base pair(s); IREBP, iron response element-binding protein; JF, Jungle Fowl; kb, kilobase(s); nt, nucleotide; RPL5, ribosomal protein L5; WL, White Leghorn; PCR, polymerase chain reaction.
may be a distinguishing characteristic of all corresponding mammalian β4GT genes.

We have established that murine somatic tissues predominantly use the 4.1-kb transcriptional start site (10). The only exception to this general pattern is found in the mid- to late pregnant and lactating mammary gland, where the 3.9-kb transcriptional start site is preferentially used. This switch to the predominant use of the 3.9-kb start site is coincident with the cellular requirement for increased β4GT enzyme levels in preparation for lactose biosynthesis. These observations, combined with a detailed promoter analysis, experimentally support a model of transcriptional regulation in which the region upstream of the 4.1-kb start site functions as a ubiquitous or housekeeping promoter for glycan biosynthesis. In contrast, the region adjacent to the 3.9-kb start site functions primarily as a mammary cell-specific promoter for lactose biosynthesis (10, 11). Based on this model, we have argued that the 3.9-kb transcriptional start site and its accompanying tissue-restricted regulatory elements have evolved in mammals to accommodate the recruited role of β4GT for lactose biosynthesis (11). One prediction of this model is that, because β4GT in nonmammalian vertebrates functions exclusively in a housekeeping (glycan biosynthesis) role, the nonmammalian gene will exhibit only one (or one set of) clustered transcriptional start site(s), characteristic of many housekeeping genes. To test this prediction and to begin to generate a data base for comparing the amino acid sequence of β4GT from diverse species to identify essential amino acids for structure-function correlations, we have isolated and characterized the β4GT gene from a nonmammalian vertebrate, the chicken.

Based on the isolation of full-length cDNA and genomic clones and on the expression of enzymatically active recombinant protein, we report that the chicken genome contains two functional, divergent β4GT genes, termed CKβ4GT-I and CKβ4GT-II, and that each encodes an α-lactalbumin-responsive β4GT homologue. CKβ4GT-I has been mapped to chicken chromosome Z in a region of evolutionary conserved synteny with the centromeric region of mouse chromosome 4 and human chromosome 9p, where β4-galactosyltransferase had previously been mapped (12, 13). Consequently, it is the CKβ4GT-I ancestral lineage that has evolved into the mammalian β4GT gene that is recognized to function in lactose biosynthesis. In contrast, CKβ4GT-II maps to chicken chromosome 8, in a region that is syntenic with human chromosome 1p, where a set of expressed sequence tags, noted to be highly similar to β4GT, have been mapped. Because both the CKβ4GT-I and CKβ4GT-II gene lineages are detectable in mammals, duplication of the ancestral β4-galactosyltransferase gene occurred over 250 million years ago in an ancestral species common to both mammals and birds.

**Materials and Methods**

**Reagents**—Restriction enzymes, reverse transcriptase, T4 DNA ligase, the Klenow fragment of DNA polymerase I, S1 nuclease, and polynucleotide kinase were from Life Technologies, Inc. or New England Biolabs. T4u DNA polymerase was from Boehringer Mannheim. Agt10 arms and λ packaging extracts were from Promega Corp. EcoRI linkers were from Collaborative Research. Radioactive nucleotides were from Amersham Corp.

**Plasmids and Cell Lines**—The chicken MSB-1 and T-249 cell lines were obtained from Dr. W. Earnshaw (Institute of Cell and Molecular Biology, University of Edinburgh, Scotland), and grown in Dubucq's modified Eagle's medium, high glucose supplemented with 10% fetal calf serum. The MSB-1 cell line is derived from a Marek's disease virus-induced lymphoma (15). The T-249 cell line was isolated from a liver tumor produced by the MC29 strain of avian leukemia virus (16). Culture media was supplemented with 100 units/ml penicillin and 50 μg/ml streptomycin. The cells were maintained at 37 °C in a 5% CO2 atmosphere.

Construction and Screening of a Chicken Hepatoma cDNA Library—Double-stranded cDNA, prepared from 10 μg of T-249 poly(A)+ RNA, was used to construct a Agt10 cDNA library using previously described procedures (6). Approximately 1 × 106 recombinant phage were screened using the bovine β4GT cDNA clone 7A as the probe. This clone contained ~1 kb of coding sequence plus ~300 bp of 3′-untranslated sequence (17).

Construction of Recombinant Transfer Vector and Recombinant Baculovirus—High Five cells (BTI-TN-5B1-4), derived from Trichoplasma ni egg cell homogenates were obtained from Invitrogen, and were grown in Hanks' TMN-FH medium (Sera-Lab Ltd, United Kingdom) supplemented with 10% fetal calf serum (Life Technologies). Linearized, wild-type Autographa californica virus DNA containing a deletion in an essential viral gene (BaculoGold DNA) was obtained from Pharmingen (San Diego, CA). The plasmid pVT-Bac was kindly donated by Dr. T. Vernet (Biotechnology Research Institute, Montreal, Canada). This transfer vector, which contains the signal peptide of the mellitin gene upstream of a multiple cloning site, was used for the construction of recombinant plasmids (18).

Plasmid pVT-Bac was digested with BamHI and bluntended using T4 DNA polymerase. A 980-bp Avai fragment from CKβ4GT-I cDNA clone 33A, which contains the coding sequence from amino acid residue 45 and includes the stop codon, was bluntened with T4 DNA polymerase and ligated into the vector. Plasmid pVT-Bac was digested with Smal and EcoR I and ligated with a 9-kb Acc1/EcoRI fragment from CKβ4GT-II cDNA clone 25B that contains the coding sequence starting from amino acid residue 36 and includes the stop codon. Each clone was sequenced across the junction point.

Recombinant baculovirus was produced as described in the BaculoGold manual supplied by the manufacturer. Infection of T. ni insect cells with the recombinant virus results in the secretion of a soluble form of each chicken polypeptide. Cleavage by the signal peptidase results in one additional amino-terminal residue (Asp) in the polypeptide encoded by clone 33A and four additional residues (Asp-Pro-Ser-Pro) in the polypeptide encoded by clone 25B.

Production of Recombinant Enzyme and Product Characterization—T. ni cells were infected at a multiplicity of infection of 5 with recombinant baculovirus. At 72 h postinfection, the medium was collected and centrifuged to remove detached cells. Galactosyltransferase assays were carried out directly on aliquots (3–10 μl) of the medium in a final reaction volume of 50 μl containing 1.25 μmol of GlcNAc as the acceptor substrate, 5 μmol of Tris-maleate buffer, pH 6.8, 1 μmol of MnCl2, 0.4 mg of Triton X-100, 200 nmol of ATP, 1 μmol of γ-galactono-1,4-lactone (Sigma), 25 μg of bovine serum albumin, and 25 nmol of UDP-Gal (1.1 Ci/mmol). The reaction mixtures were incubated at 37 °C for 15 min, and the product was isolated by ion exchange chromatography and quantified as described previously (19).

An aliquot of the radioactive product was subjected to high pH anion exchange chromatography with pulsed amperometric detection. The system consisted of a Dionex Bio-LC gradient pump, a Carbopac-100 column (4 × 250 mm), and a model PAD 2 detector. The following pulse potentials were used for detection: E1 = 480 mV; E2 = 0.60 V (t1 = 120 ms); E3 = 0.60 V (t2 = 60 ms). Samples were dissolved in 0.1 M NaOH, and the column was eluted isocratically with 0.1 M NaOH for 10 min, after which a gradient was applied that increased the concentration of sodium acetate to 0.1 M NaOH by 2.5 mM/min. The flow rate was 1 ml/min. The eluate was collected in 0.5-ml fractions, and radioactivity was counted in the individual fractions. The elution position of the radioactive product was compared with that of the reference compounds: Gal, Galβ1,4GlcNAc, Galβ1,3GlcNAc, and Galβ1,4Glc, as determined by pulsed amperometric detection.

The effect of α-lactalbumin on the acceptor preference of each recombinant enzyme was evaluated by performing the standard galactosyltransferase assay as described above, using either GlcNAc or Gal as the acceptor, in the presence of increasing α-lactalbumin concentrations (0.1–2.0 mg/ml).
Northern and Southern Blot Analyses—RNA and DNA were isolated from T-249 cells by the guanidine isothiocyanate method of Chirgwin et al. (21). RNA was also isolated from MSB-1 cells and various tissues of a young female White Leghorn chicken. The isolation of poly(A)\(^+\) RNA and the Northern and Southern blot analyses were carried out as described previously (17).

S1 Nuclease Analysis—S1 nuclease protection assays were performed as described previously (6). A 656-bp NotI-NorI CK\(\beta\)4GT-I genomic DNA fragment was isolated that flanked the anticipated transcriptional start site(s). The NorI cleavage site corresponded to nt +227 in Fig. 1A. A single-stranded probe complementary to the CK\(\beta\)4GT-I transcribed sequence was prepared by sequencing extension of an M13mp18 clone in the presence of \(^{32}\)PdATP and Klenow polymerase. The probe contained an additional 87 bp of polylinker sequence. A 305-bp NorI CK\(\beta\)4GT-II genomic DNA fragment was isolated that spanned the 5'-end of the CK\(\beta\)4GT-II sequence and a single-stranded probe, containing 97 bp of polylinker sequence, was prepared as described above.

After purification, probe hybridization to MSB-1 and T-249 RNA was carried out at 62°C overnight. The samples were digested with S1 nuclease, and the products were analyzed on a 7% polyacrylamide, 8 M urea gel.

DNA Sequence and Computer Analyses—Double-stranded, dyeoxyDNA sequencing was performed using the Sequenase kit from U.S. Biochemical Corp. The M13, T7, and T3 primers as well as synthetic oligonucleotides used as sequencing primers, oligonucleotides were synthesized by the Johns Hopkins Core Facility. Sequences were analyzed using MacVector from International Biotechnologies, Inc. or the GeneWorks Nucleic Acid and Protein Sequencing Analysis program from IntelliGenetics, Inc.\(^2\)

Oligonucleotide Primers—The oligonucleotide primers used to determine the intron/exon boundaries for the CK\(\beta\)4GT-I genomic clone were as follows: EX 1F, 5'-CCCGGACCACCGTCTCCGACA-3'; EX 2F, 5'-TACATGGCAACCAATTCTCTCA-3'; EX 2R, 5'-GACCTCAGGTTTGTGTGC-3'; EX 3F, 5'-TACAATGTCATACCGCACAAC-3'; EX 3R, 5'-AGGTTCCGGTATCCTCACA-3'; EX 4F, 5'-AAAGTCAATTGGGTTCCTCC-3'; EX 4R, 5'-TGTCACAAGCCGACACACTCC-3'; EX 5F, 5'-TGGGAAATTGCAAGATGATT-3'; EX 5R, 5'-TCTGCTATTTCCCAATGACAG-3'; EX 6R, 5'-AGGAGGTCCAGGACACATGCA-3'.

Localization of CK\(\beta\)4GT-I and CK\(\beta\)4GT-II in the Chicken Genome—Each CK\(\beta\)4GT gene was mapped using a mismatched primer PCR approach based on nucleotide substitutions found in either the Jungle Fowl (JF) or White Leghorn (WL) product (25). To map the CK\(\beta\)4GT genes, DNA from 52 progeny of the East Lansing reference population (JF × WL) × WL were used to follow segregation of the JF allele. When bovine sequences are located at Asn 50 Asn59, Asn 64, Asn 87, and Asn 358. The

RESULTS

Isolation and Characterization of Two Chicken Homologues of \(\beta\)4GT—The strategy we used to clone chicken \(\beta\)4GT was to construct a \(\beta\)gt10 nonexpression cDNA library using poly(A)\(^+\) RNA isolated from the T-249 cell line and to screen it with our bovine \(\beta\)4GT cDNA probe. Our choice of T-249 cells for library construction and the bovine probe for library screening was based on two considerations. First, by direct enzymatic assay, T-249 cells exhibited a 3-fold higher level of \(\beta\)4GT activity compared with MSB-1 cells. Second, Northern blot analysis of T-249 poly(A)\(^+\) RNA revealed a broad hybridization positive band of ~2.5 kb using the bovine \(\beta\)4GT probe. This latter result indicated that there was sufficient similarity in the nucleotide sequence to permit direct screening with the bovine probe.

Approximately 1 × 10\(^6\) independent recombinants were subsequently screened, resulting in the isolation of 18 cDNA clones. The six largest inserts were subcloned and partially sequenced. This preliminary analysis, in combination with partial restriction endonuclease mapping of the other 12 isolates, revealed the presence of two distinct groups of clones, CK\(\beta\)4GT-I (nine clones) and CK\(\beta\)4GT-II (nine clones).

Nucleotide Sequence and Translated Amino Acid Sequence of CK\(\beta\)4GT-I—The complete nucleotide sequence of clone 33A is shown in Fig. 1A. The first in-frame ATG encoding a long open reading frame (located at nt +1 to +3) was present in a sequence context appropriate for translation initiation (23) and therefore was designated the initiating Met. The entire 3'-untranslated region (1137 bp) is present in this clone, since a consensus polyadenylation signal (ATTAAA) is present 15–20 bp upstream of a 21-nucleotide tail.

As discussed below, the additional 5'-untranslated sequence (~17 to ~210), shown in Fig. 1A, was subsequently obtained after carrying out S1 analysis on a fragment derived from an appropriate genomic clone.

The coding region of clone 33A is 66% identical at the nucleotide level, and 62% at the amino acid level, to the corresponding bovine sequence. Translation predicts a type II, membrane-bound, potentially glycosylated protein of 362 amino acids with an NH\(_2\)-terminal cytoplasmic domain of 16 amino acids, a single transmembrane domain of 20 amino acids (assuming that the Gly residue at position 36 defines the COOH-terminal boundary of this domain), a stem region of ~55 amino acids, and a COOH-terminal domain of 271 amino acids. One N-linked glycosylation consensus site is located at Asn\(^56\). The length of this chicken \(\beta\)4GT homologue is 40 amino acids shorter than the predicted long protein isoform of bovine \(\beta\)4GT due to multiple small deletions in the stem region combined with a cytoplasmic domain that is eight amino acids shorter.

Nucleotide Sequence and Translated Amino Acid Sequence of CK\(\beta\)4GT-II—The complete nucleotide sequence of CK\(\beta\)4GT-II (clone 25B) is shown in Fig. 1B. The first in-frame ATG codon of the longest open reading frame (located at nt +1 to +3) was assigned as the initiating Met, based on Kozak's rules for translation initiation (24) and the fact that an upstream in-frame termination codon (TGA) is present at nt ~117 to ~115. Consequently, this cDNA clone contains 209 bp of 5'-untranslated sequence plus a coding sequence of 1119 bp. The complete 3'-untranslated sequence (1100 bp) is also present in this clone, since a consensus polyadenylation signal (AATAAA) was located 17–22 bp upstream of a 65-nucleotide tail.

The coding region of CK\(\beta\)4GT-II is 59% identical at the nucleotide level, and 49% identical at the amino acid level, to the corresponding bovine sequence. Translation predicts a type II, membrane-bound, potentially glycosylated protein of 373 amino acids with an NH\(_2\)-terminal cytoplasmic domain of 15 amino acids, a single transmembrane domain of 18 amino acids, a stem region of ~64 amino acids, and a COOH-terminal catalytic domain of 276 amino acids. Five N-linked glycosylation consensus sites are located at Asn\(^50\) Aan\(^59\), Asn\(^64\), Asn\(^87\), and Aan\(^358\).
length of this chicken β4GT homologue is 29 amino acids shorter than the predicted long protein isoform of bovine β4GT due to multiple deletions in the stem region, a cytoplasmic domain that is shorter by nine amino acids, and a six-amino acid extension at the COOH terminus.

At the nucleotide level, the coding region of CKβ4GT-I and CKβ4GT-II is 61% identical, the 3'-untranslated region of each transcript was used to probe the Northern blot. As indicated above, these regions share no sequence identity.

The CKβ4GT-I probe identifies an mRNA species of ~2.5 kb (Fig. 2A, lane 1). The minor band at ~4.3 kb is due to nonspecific hybridization. The CKβ4GT-II probe hybridizes to a band of ~3.5 kb (Fig. 2B, lane 1) and a ~5.5 kb band (Fig. 2B, lane 2). These bands may represent the full-length transcript and an alternatively spliced variant, respectively.

Two Functional Chicken β4-Galactosyltransferase Genes

Northern Blot Analysis—Northern blot analysis was carried out using RNA isolated from the T-249 cell line, used for cDNA library construction, to determine the size and number of transcripts corresponding to each cDNA clone. Since the nucleotide sequence of the coding region of CKβ4GT-I and CKβ4GT-II is 61% identical, the 3'-untranslated region of each transcript was used to probe the Northern blot. As indicated above, these regions share no sequence identity.

The CKβ4GT-I probe identifies an mRNA species of ~2.5 kb (Fig. 2A, lane 1). The minor band at ~4.3 kb is due to nonspec-
cific hybridization of the probe to 28 S ribosomal RNA. Since CKβ4GT-I clone 33A (−2.2 kb) contains a consensus polyadenylation site and a poly(A) tail, the missing sequence (estimated to be ~150 bp) is from the 5'-end. The CKβ4GT-I probe was subsequently removed from the Northern blot, which was then hybridized with the 3'-untranslated region of the CKβ4GT-II clone. A single transcript of ~2.5 kb was detected, suggesting that clone 25B represents the full-length transcript (Fig. 2A, lane 2).

**CKβ4GT-I and CKβ4GT-II Each Encode an Enzymatically Active, α-Lactalbumin-responsive β4-Galactosyltransferase—** To determine if each chicken cDNA encodes a β1,4-galactosyltransferase, constructs were assembled that fused the luminal domain of either CKβ4GT-I or CKβ4GT-II to the signal sequence in the stem region of mellitin. Expression of each cDNA in T. ni insect cells resulted in the secretion of enzymatically active, soluble enzyme. As shown in Table 1, both CKβ4GT-I and CKβ4GT-II showed a relatively high galactosyltransferase activity using UDP-Gal as the donor and GlcNAc as the acceptor substrate. Furthermore, each recombinant galactosyltransferase is able to interact productively with α-lactalbumin as evidenced by the production of lactose. When UDP-Gal was replaced by equal concentrations of UDP-GalNAc, UDP-GlcNAc, or UDP-Glc, the activity was reduced to less than 1% of that measured with UDP-Gal. This low level of residual activity was comparable with that observed with affinity-purified bovine β1,4-galactosyltransferase (data not shown).

Since both β1,3- and β1,4-galactosyltransferases have been detected in chicken, the product formed using GlcNAc as the acceptor was characterized. On high pH anion exchange chromatography, the radioactive product migrated as a single peak, whose elution position corresponded to authentic Galβ1,4GlcNAc (retention time 7.2 min). No radioactivity was detected at the elution position of Galβ1,3GlcNAc (retention time 10.5 min). Collectively, these results establish that both CKβ4GT-I and CKβ4GT-II encode an α-lactalbumin-responsive UDP-Gal:GlcNAc-R β1,4-galactosyltransferase.

**Comparison of the Amino Acid Sequences of CKβ4GT-I, CKβ4GT-II, and Bovine β4GT—** The protein domain structure established for the cloned mammalian β4-galactosyltransferases consists of (i) a short NH2-terminal cytoplasmic domain of 11 or 24 amino acids depending on the protein isoform (6) and (ii) a large COOH-terminal luminal domain (~224 amino acids) containing the catalytic center, linked to a single transmembrane domain (20 amino acids) through a potentially glycosylated peptide segment of ~85 amino acids, termed the stem region. The catalytic domain can be further subdivided into two distinct structure/function subdomains. (i) The NH2-terminal region of the catalytic domain contains a 113-amino acid loop formed by the only intramolecular disulfide bond present in the protein, between Cys134 and Cys247 (Ref. 25; see bovine sequence in Fig. 3, solid arrowheads). This loop plus adjacent sequence in the stem region (the stem region is defined as the amino acid sequence between the transmembrane domain and Cys134) is involved in α-lactalbumin binding as established by protection studies (26) and antibody blocking studies (27, 28). (ii) The COOH-terminal 155-amino acid segment contains two polypeptides, in the vicinity of Cys242 (Fig. 3, bovine sequence), that can be affinity-labeled with UDP-Gal analogues (26, 29) or have been implicated in substrate binding by site-directed mutagenesis (29).

In the context of this domain structure, a comparison of the amino acid sequence between each chicken β4GT homologue and a mammalian (bovine) β4GT is interesting (Fig. 3). The amino acid sequence of CKβ4GT-I and CKβ4GT-II is 62 and 49% identical, respectively, to the bovine β4GT sequence and only 52% identical to each other. Thus, CKβ4GT-I and CKβ4GT-II are as divergent from each other as they are from their mammalian counterpart. When the amino acid sequences of all three proteins are compared, the sequence identity is reduced to about 42%. The structural domains that are least conserved are the stem domain and the NH2-terminal region of the cytoplasmic domain (Fig. 3). Of particular note, six of the seven Cys residues included in the two Cys residues involved in intramolecular disulfide bond formation (Cys3134 and Cys247 in the bovine sequence, Fig. 3) are conserved in the CKβ4GT-I and CKβ4GT-II sequences. The remaining Cys residue (Fig. 3, open arrowhead) is conserved only in CKβ4GT-I; in CKβ4GT-II, this Cys residue is replaced by Tyr. As discussed below, this fortuitous Cys to Tyr replacement is a useful marker to follow the evolutionary gene lineage of CKβ4GT-I and CKβ4GT-II in the human and mouse genomes.

**Southern Blot Analysis and Isolation of CKβ4GT-I and CKβ4GT-II Genomic Clones—** The comparison of the nucleotide and amino acid sequence suggested to us that each chicken homologue is encoded by a separate nonallelic gene that arose as a consequence of duplication of an ancestral gene followed by Table I: Expression of enzymatically active recombinant CKβ4GT-I and CKβ4GT-II in T. ni insect cells

|                | Transfer to GlcNAc | Transfer to Gla | Transfer to Glu |
|----------------|--------------------|----------------|-----------------|
| CKβ4GT-I       | 153.0 (100%)       | 25.0 (16%)     | 3.9 (2%)        |
| CKβ4GT-II      | 28.0 (100%)        | 3.4 (12%)      | 36.4 (130%)     |
| α-LA (−)       | 132.0 (86%)        | 132.0 (86%)    |                 |
| α-LA (+)       | 9.0 (45%)          | 9.0 (45%)      |                 |
To establish the intron/exon boundaries, subcloned fragments of the genomic clones were sequenced using exon-specific oligonucleotide primers. Since intron/exon boundaries within the protein coding region are generally conserved across species lines, the CKβ4GT-I and CKβ4GT- II exon-specific primers used for sequencing were chosen based on the intron/exon boundaries established for the murine (30) and human β4GT gene (9). The sequence at the intron/exon boundaries determined for CKβ4GT-I and CKβ4GT-II along with the corresponding murine sequence is shown in Table II. Based on this analysis, it is clear that the two chicken β4GT genes share identical intron/exon boundaries with each other and their mammalian homologues, an observation that supports the notion of a gene duplication of an ancestral gene. However, unlike the mammalian β4GT gene, in which the entire 5′-untranslated region and first 415 bp of coding sequence are present on exon 1, the CKβ4GT-II gene has one intron within its 5′-untranslated region, positioned at nt 45.

Expression of CKβ4GT-I and CKβ4GT-II in Various Adult Chicken Tissues—The presence of two functional β4GT genes in the chicken would suggest that each is regulated in a tissue-specific manner. To examine this possibility, a Northern blot containing poly(A) RNA isolated from brain, kidney, liver, lung, spleen, and pancreas of a female adult chicken was prepared and hybridized sequentially, with a probe derived from the 3′-untranslated region of each clone. As seen in Fig. 5, the steady state levels of the CKβ4GT-II mRNA are significantly higher in the panel of somatic tissues examined. Somewhat surprisingly, transcript levels are also high in the brain; this is in contrast to mice and humans, where β4GT mRNA levels are about 10-fold lower in the brain as compared with other somatic tissues.

| CKβ4GT-I | MKEPALLLLPRLYVNLNLSSYATLPDTGALQSLAC----41 |
| CKβ4GT-II | MTRLLLAPRKHLLYVNLNLSSYATLPDTGALQSLAC----40 |
| BOVS4GT | MKEPULLGQAAMSIHLLYVNLNLSSYATLPDTGALQSLAC----50 |
| CKβ4GT-I | PP----PEEP----PEEPFANLSSP---- R----PRPP----AAAR----73 |
| CKβ4GT-II | DFFYFBFRHMRVAYVPLFSSS---- DTF--YPCDCA----77 |
| BOVS4GT | PQCrVuvecFQQSCHGAAAGTSEGLSLEQVA---SPPQNSNQFAQPS100 |
| CK4GT-I | NKNLKVFQSLARKFLKVAQPLPKKFLHMKWYK----159 |
| CK4GT-II | NKLKVFQSLARKFLKVAQPLPKKFLHMKWYK----164 |
| BOVS4GT | NDALCFQQKLARKFLKVAQPLPKKFLHMKWYK----200 |
| CK4GT-I | DIPFLKLKLCYGLK----DDEPERAKLLMEGALSYD----268 |
| CK4GT-II | DIPFLKLKLCYGLK----DDEPERAKLLMEGALSYD----214 |
| BOVS4GT | DIPFLKLKLCYGLK----DDEPERAKLLMEGALSYD----249 |
| CK4GT-I | PSSDVLPLLKFRLYQPEYKSLQDQVPGCST-----258 |
| CK4GT-II | PSSDVLPLLKFRLYQPEYKSLQDQVPGCST-----264 |
| BOVS4GT | PSSDVLPLLKFRLYQPEYKSLQDQVPGCST-----299 |
| CK4GT-I | QKKN1FWFVWCMGCGDEDSNLLFZLVLKSR1--131 |
| CK4GT-II | QKKN1FWFVWCMGCGDEDSNLLFZLVLKSR1--134 |
| BOVS4GT | QKKN1FWFVWCMGCGDEDSNLLFZLVLKSR1--349 |
| CK4GT-I | MQGPNKAVULLKFLKVAQPLPKKFLHMKWYK----358 |
| CK4GT-II | MQGPNKAVULLKFLKVAQPLPKKFLHMKWYK----364 |
| BOVS4GT | MQGPNKAVULLKFLKVAQPLPKKFLHMKWYK----399 |
| CKEA4GT-I | A---E---A---342 |
| CKEB4GT-II | H----RPRPLARG----273 |
| BOVS4GT | H----402 |
A comparison of the respective coding regions of the two chicken β4GT genes with their mammalian counterpart suggests an answer to the first question. As previously discussed (Fig. 3), the coding sequence of CKβ4GT-I, at the nucleotide level, exhibits a somewhat greater sequence identity (66%) to the mammalian (bovine) gene compared with CKβ4GT-II (59%). Based on this analysis, it would appear that the CKβ4GT-I lineage gave rise to the well characterized mammalian β4GT gene that was recruited for lactose biosynthesis.

However, a more definitive approach, based on chromosomal assignment, can be used to trace the fate of each chicken β4GT gene lineage in the mammalian (human) genome. This strategy takes advantage of the comparative gene maps that have been established between different species, revealing regions of conserved synteny. These regions define groups of genes that are located together in close proximity on a chromosome. Therefore, given the location of a gene in one species, the location in another can be predicted.

Human β4GT maps to chromosome 9p13 (13) and to the centromeric region of mouse chromosome 4 (12) in a region that shows conserved synteny with aconitase. Aconitase also has a second function, in that it acts as the iron response element-binding protein (IREBP) (31). In the chicken, aconitase I/IREBP has been mapped to chromosome Z (32). Using allelespecific primers, we found that CKβ4GT-I maps to chicken chromosome Z to a region within 2 centimorgans of aconitase I/IREBP (Fig. 6A). This assignment, then, unequivocally establishes that the CKβ4GT-I gene lineage gave rise to the previously characterized human and mouse β4GT gene.

We have also mapped the CKβ4GT-II gene to chicken chromosome 8. Ribosomal protein L5 (RPL5) also maps to this small chromosome (33). In the human genome, RPL5 maps to chromosome 1p31–32 (Fig. 6B). 3 We have been able to take advantage of the recently established human gene map of expressed sequence tags (UniGene data base (22) to determine if any sequence tags, with noted similarity to β4GT, are present on human chromosome 1p31–32 near RPL5. In fact, a group of 10 sequence tags (e.g. accession numbers W07207 and AA453005), which delineate a partial mRNA of ~1.5 kb, have been mapped to this chromosomal region (Fig. 6B). This ~1.5-kb mRNA has an open reading frame that encodes a protein of 279 amino acids that corresponds to about 75% of the coding sequence (based on the CKβ4GT-II coding sequence), including the complete catalytic domain. At the nucleotide and amino acid level, ...

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3 N. Kenmochi, T. S. Kawaguchi, S. Rozen, E. Davis, N. Goodman, T. Tanaka, and D. C. Page, manuscript in preparation.
these two gene occurred after divergence, it would be anticipated that dependent divergence. When did duplication of the ancestral "b" still encode an enzymatically active, head sive b sequence, suggestive of an UDP-galactose:N-acetylglucosamine b-lactalbumin-responsive, enzymatically active pro- teins that are only 52% identical (Fig. 3). Based on the consen- sus that the relative positions of the six cystinyl residues in the catalytic domain and the essential amino acids in the two polypeptides pointed out above that have been affinity-labeled with UDP-Gal analogues are present (26, 27). Last, at the position of the Cys342 to Tyr substitution (Fig. 3, open arrowhead), which distinguishes the respective CK4Gt-I and CK4Gt-II gene lineages, a Tyr is present. The open question is whether this human CK4Gt-II homologue on chromosome 1p still encodes an enzymatically active, α-lactalbumin-responsive β4-galactosyltransferase. DISCUSSION The unanticipated result from this study was the demonstration that the chicken genome contains two functional, nonal- lelic β4GT genes (CK4Gt-I and CK4Gt-II), which encode distinct α-lactalbumin-responsive, enzymatically active proteins that are only 52% identical (Fig. 3). Based on the conserva- tion of the intron/exon boundaries within the coding region among CK4Gt-I, CK4Gt-II, and the mammalian β4GT genes, it is clear that these chicken β4GT genes arose as the consequence of duplication of an ancestral gene and subsequent divergence. When did duplication of the ancestral "b" gene occur, relative to the independent evolution of mammals and birds? In considering this question, it is essential to recall that current opinion holds that mammals and birds last shared a common ancestor ~250 million years ago. Consequently, depending on the time of the gene duplication, relative to the divergence from their common ancestor, two different outcomes can be predicted. First, if duplication of the avian β4GT gene occurred after divergence, it would be anticipated that these two β4GT genes would be a distinguishing characteristic of the avian genome and would not be found in the mammalian genome. In contrast, if the gene duplication took place prior to the separation of mammals and birds from their common pred- cessor, then one would anticipate finding both the CK4Gt-I and CK4Gt-II gene lineages in the mammalian genome. As summarized in Fig. 6, we have mapped the CK4Gt-I gene to chromosome Z, in a region that is syntenic with human chromosome 9p13, which is the chromosomal location of human β4GT. Consequently, it can be concluded that it was the CK4Gt-I gene lineage that was recruited for lactose biosynthesis during the evolution of mammals. Additionally, CK4Gt-II maps to chicken chromosome 8, in a region that is syntenic with human chromosome 1p, and where a set of expressed sequence tags with noted similarity to β4GT has recently been mapped. Thus, both the CK4Gt-I and CK4Gt-II lineages can be detected in the mammalian genome, indicating that duplication of the ancestral β4GT gene occurred at least 250 million years ago, prior to the divergence of mammals and avians from their common ancestor. The Human Genome Contains Additional CK4Gt-II-related Genes—Interestingly, four additional sets of expressed sequence tags with noted similarity to β4GT were also noted in the UniGene data base. Three sets map to human chromosomes 1q21-23, 3q13, and 18q11, respectively. The fourth set of expressed sequence tags has not yet been assigned a chromo- somal position. From the available coding sequence, it is clear that three of these additional human β4GT-related genes encode a type II protein with a coding sequence that is ~40% identical with each other and with mouse or human β4GT.2 (For the fourth set of sequence tags, mapped to 18q11, only the C-terminal 120 amino acids have been reported). An inspection of their primary sequence reveals that the relative positions of the six cystinyl residues are also conserved. Last, at the position of the Cys342 to Tyr substitution (Fig. 3, open arrowhead), which distinguishes the respective CK4Gt-I and CK4Gt-II lineages, a diagnostic Tyr is present in each of the additional homologues. Consequently, it would appear that these four additional human β4GT homologues have arisen from multiple duplications within the CK4Gt-II gene lineage. Multiple mouse expressed sequence tags with noted similarity to β4GT have also been deposited in the dbEST data base. Unfortunately, these mouse sequence tags have not been mapped; consequently, it is not possible to group these clones

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For a detailed overview of phylogeny, refer to The Tree of Life site on the World Wide Web at http://phylogeny.arizona.edu/tree/phylogeny.html.
flanking the transmembrane domain may also be required for a fully functional retention signal. Interestingly, a comparison of the amino acid sequence of the NH2-terminal regions of a group of resident proteins with a similar Golgi distribution has failed to reveal a sequence motif in common that could function as a Golgi retention signal.

An alternative approach to identifying essential amino acids within a functional domain is to compare the primary structure of the same protein from evolutionarily distant species. Using this strategy, one can establish the "mutations" allowed by nature consistent with maintenance of the functional domain. This approach is particularly applicable for an interspecies comparison of β4GT because the NH2-terminal region including the lumenal stem domain (amino acids 1–92), in contrast to the COOH-terminal catalytic domain, exhibits the greatest divergence in primary structure (Fig. 3). Interestingly, within this region of divergence, the transmembrane domain and the nine amino acids of the cytoplasmic domain that immediately flank the transmembrane domain, stand out as being highly conserved. This point is further amplified by an inspection of the sequence alignment of the NH2-terminal regions of the human, bovine, murine, CKβ4GT-I, and CKβ4GT-II β4GT polypeptides (Fig. 7). In the NH2-terminal flanking sequence, four amino acids are identical and three are conservative replacements. Within the transmembrane domain, four amino acids are identical and six are conservative replacements. In contrast, the amino acids in the remainder of the cytoplasmic domain and the lumenal sequence flanking the transmembrane domain are not conserved. The fact that the indicated subset of amino acids distributed within the transmembrane and cytoplasmic domain have remained conserved over ~250 million years of evolution suggests that they may serve as a "functional unit" for retention of β4GT in the trans-Golgi.

Absence of the 13-Amino Acid NH2-terminal Extension in the Chicken β4GT Homologues—Transcription of the murine β4GT gene in somatic tissues takes place at one of two different start sites that are separated by ~200 bp (Fig. 8). Use of these two transcriptional start sites results in a 4.1- and a 3.9-kb mRNA. The main difference between these two mRNAs is the length and extent of the predicted secondary structure of the respective 5'-untranslated regions (10). The 4.1-kb start site is positioned upstream of the first two in-frame ATGs, whereas the 3.9-kb start site is located between these two in-frame ATGs (Fig. 8). Consequently, translation of the 4.1- and 3.9-kb mRNAs results in the biosynthesis of two protein isoforms that differ only in the length of their respective NH2-terminal cytoplasmic domains. The "long" and "short" β4GT protein isoforms have NH2-terminal cytoplasmic domains of 24 and 11 amino acids, respectively.

The functional significance of this additional 13 amino acids has been the subject of much interest and speculation. Based on the conclusions from a number of investigators who showed that the transmembrane domain of β4GT is sufficient to retain a reporter protein in the Golgi compartment (reviewed in Refs. 36–38) and our demonstration that both β4GT protein isoforms were localized in the trans-Golgi compartment as assessed by immunoelectron microscopy, we have concluded that both isoforms are functionally equivalent Golgi-resident proteins (39). A contrasting viewpoint has been put forth by Shur and colleagues (40), who suggested that the 13-amino acid extension serves a functional role by overriding the trans-Golgi retention signal, thereby directing a small percentage or "portion" of this isoform to the cell surface. It was posited that, at the cell surface, the long β4GT isoform functions as a cell adhesion molecule by virtue of its ability to interact with the cytoskeleton via this 13-amino acid extension (41).
increased transcription from the 3.9-kb start site. In step two, whereas in CK
b
indicate the position of the second in-frame Met in the mammalian

promoter analysis of the murine

the functional role for the 13-amino acid extension that distin-

long

the 13-amino acid extension characteristic of the mammalian

Note the subset of conserved amino acids distributed within the transmembrane and cytoplasmic domain, which may serve as a “functional unit” for retention of β4GT in the trans-Golgi. Also note that the NH2-terminal 13 amino acids, which have been proposed to override the trans-Golgi retention signal and thereby direct some of the long mammalian β4GT protein isoform to the cell surface (40), are absent in both chicken β4GT homologues. Although it had been reported that the cytoplasmic domain of the human sequence lacks the Ser residue at amino acid 11 (8), when

the two-step mechanism to generate the elevated levels of

specific cytoplasmic domains, two features stand out. First,

that, because

pointed out in the Introduction, a prediction of this model is

In the context of the biological significance of this 13-amino acid extension, a comparison of the cytoplasmic domains of the mammalian and the two chicken β4GT proteins is instructive. Since β4GT has been reported on the cell surface of a variety of chicken cells and tissues (see Ref. 42 and references therein), one would anticipate that a functional domain responsible for the redirection of a protein from the Golgi to the cell surface would be conserved between mammals and chickens.

From an inspection of the amino acid sequences of the respective cytoplasmic domains, two features stand out. First, the 13-amino acid extension characteristic of the mammalian long β4GT protein isoform is absent in both chicken β4GT homologues. Second, in place of this 13-amino acid extension, a tetra- or pentapeptide is present, which with the exception of the initiating Met, does not have any sequence in common with the mammalian NH2-terminal extension. The lack of conservation in the amino acid sequence of the cytoplasmic domains between the two chicken and the mammalian β4-galactosyltransferases needs to be taken into account when considering the functional role for the 13-amino acid extension that distinguishes the long β4GT protein isoform in mammals.

In Contrast to the Murine β4GT Gene, Transcription of the CKβ4GT-I Gene Takes Place at a Single Start Site—Based on a detailed promoter analysis of the murine β4GT gene, we have provided a biological and functional rationale for the unusual structure of the 5′-end of this glycosyltransferase gene (Fig. 8). Specifically, we have proposed a model of transcriptional and translational regulation in which the region upstream of the 4.1-kb start site functions as a ubiquitous or housekeeping promoter for glycan biosynthesis. In contrast, the region adjacent to the 3.9-kb start site functions primarily as a mammary cell-specific promoter for lactose biosynthesis (10, 11). The essential feature of our model is that mammals have evolved a two-step mechanism to generate the elevated levels of β4GT enzymatic activity, in the lactating mammary gland, that are required for lactose biosynthesis. In step one, there is an up-regulation of the steady state levels of β4GT mRNA, due to increased transcription from the 3.9-kb start site. In step two, the 3.9-kb β4GT transcript is translated more efficiently, relative to its housekeeping counterpart, due to deletion of most (−200 nt) of the long GC-rich 5′-untranslated sequence characteristic of the 4.1-kb mRNA.

Based on this model, we have argued that the 3.9-kb transcriptional start site and its accompanying tissue-restricted regulatory elements have evolved in mammals to accommodate the recruited role of β4GT for lactose biosynthesis (11). As pointed out in the Introduction, a prediction of this model is that, because β4GT in nonmammalian vertebrates functions exclusively in a housekeeping (glycan biosynthesis) role, the gene will exhibit only one (or one set of) clustered transcriptional start site(s), characteristic of many housekeeping genes. The structures of the respective 5′-end of the CKβ4GT-I and the murine β4GT gene are summarized in Fig. 8. Note that in contrast to the mammalian β4GT gene, the CKβ4GT-I gene has a single transcriptional and translational start site, which is used preferentially in the lactating mammary gland, along with its accompanying tissue-restricted regulatory elements, have evolved in mammals to accommodate the recruited role of β4GT for lactose biosynthesis (11).

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