The LECl1 Chinese hamster ovari (CHO) gain-of-function mutant expresses an α1,3fucosyltransferase (α1,3Fuc-T) activity that generates the LeX, sialyl-LeX, and VIM-2 glycan determinants and has been extensively used for studies of E-selectin ligand specificity. In order to identify regulatory mechanisms that control α1,3Fuc-T expression in mammals, mechanisms of FUT gene expression were investigated in LECl1 cells and two new, independent mutants, LECl1A and LECl1B. Northern and ribonuclease protection analyses, using probes that span the coding region of a cloned CHO FUT gene, detected transcripts in each LECl1 mutant but not in CHO cells or other gain-of-function CHO mutants that express a different α1,3Fuc-T activity. Coding region sequence analysis and α1,3Fuc-T acceptor specificity comparisons with recombinant hamster Fuc-Tv and Fuc-TV showed that the cloned FUT gene is orthologous to the human FUT6 gene. Southern analyses identified two closely related FUT6 genes in the Chinese hamster, whose evolutionary relationships are discussed. The blots showed that rearrangements had occurred in LECl1A and LECl1 genomic DNA, consistent with a cis mechanism of FUT6 gene activation in these mutants. By contrast, somatic cell hybrid analyses revealed that LECl1B cells express FUT6 gene transcripts due to the loss of a trans-acting, negative regulatory factor. Sequencing of reverse transcriptase-polymerase chain reaction products identified unique 5′- and 3′-untranslated region sequences in FUT6 gene transcripts from each LECl1 mutant. Northern and Southern analyses with gene-specific probes showed that LECl1A cells express only the cgFUT6A gene (where cg is Cricetulus griseus), whereas LECl1B cells express only the cgFUT6B gene. In LECl1A × LECl1B hybrid cells, the cgFUT6A gene was predominantly expressed, as predicted if a trans-acting negative regulatory factor functions to suppress cgFUT6B gene expression in CHO cells. This factor is predicted to be a cell type-specific regulator of FUT6 gene expression in mammals.

α1,3Fucosyltransferases (α1,3Fuc-T) transfer fucose to lactosamine sequences in glycan units, thereby creating oncofetal antigens that may function as cell recognition determinants (reviewed in Refs. 1–3). Because fucose is added last in this synthesis, regulated expression of an α1,3Fuc-T activity may be critical to controlling a specific cell-cell adhesion event. This principle was nicely demonstrated in mice by targeted disruption of the FUT7 gene (4). Mice lacking Fuc-TVII exhibit an increase in circulating lymphocytes, neutrophils, monocytes, and eosinophils that rely on ligands fucosylated by Fuc-TVII to bind to selectins on vascular endothelium. Leukocytes lacking Fuc-TVII are also defective in extravasation from the bloodstream following an inflammatory stimulus, and they home poorly to spleen and lymph nodes (4).

There are five known human FUT genes that encode an α1,3Fuc-T activity (reviewed in Refs. 2 and 3). The FUT3, FUT5, and FUT6 (Lewis) genes reside in a cluster on chromosome 19 (5, 6); the FUT4 gene is on chromosome 11 (7), and the FUT7 gene is on chromosome 9 (5). The recently described cDNA encoding mouse Fuc-TIX identifies an additional FUT locus (8). The transferases encoded by these FUT genes transfer fucose to GlcNAc in lactosamine units to generate the LeX and/or sialyl-LeX determinants. Fuc-TIII generates in addition the LeA, LeB, sialyl-LeA, and sialyl-LeB determinants (9). The human α1,3Fuc-T activities are differentially expressed in adult tissues and in cancer (2, 10, 11). The enhanced ability of cancer cells to express sialyl-LeX has been suggested to aid in their growth and metastatic properties and to correlate with poor prognosis (reviewed in Ref. 12). Consistent with this is the finding that P-selectin-deficient mice exhibit increased experimental metastasis of human colon carcinoma cells (13). Therefore, it is important to identify factors that control the expression of the FUT genes and thereby regulate α1,3Fuc-T levels and activities in a cell type- or tissue-specific fashion. To date it...
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is known that the 5'-untranslated regions of the FUT3, FUT5, and FUT6 genes are complex and that different transcripts arise from differential splicing (11), but promoter regions of these and related genes have not been isolated.

Gain-of-function mutants that express an α(1,3)Fuc-T activity not detectable in wild-type cells provide an approach to identifying factors that serve to regulate FUT gene expression in vivo (14). The LEC11 CHO mutant expresses an α(1,3)Fuc-T activity that generates the LeX, sialyl-LeX, and VIM-2 determinants on cell-surface glycans (15–17). The sialyl-LeX determinant is instrumental in causing LEC11 cells to be recognized by E-selectin (18–21). By contrast, parent CHO cells have no α(1,3)Fuc-T activity and do not bind antibodies that recognize Lewis antigens, nor do they bind E-selectin expressed on activated endothelial cells (15–21).

Since the de novo expression of an α(1,3)Fuc-T activity in gain-of-function LEC11 CHO mutants provides an approach to identifying regulatory mechanisms that operate in vivo, we investigated the molecular basis of α(1,3)Fuc-T gene expression in three independent LEC11 mutants. We show in this paper that each LEC11 mutant expresses one of two Chinese hamster (Cricetulus griseus; cg) FUT genes that are both orthologous to the human FUT6 gene, whereas CHO cells contain no cgFUT6 gene transcripts by RNase protection analysis. Investigations of somatic cell hybrids formed between LEC11 mutants and CHO cells, as well as LEC11 mutants with each other and with other gain-of-function mutants, show that LEC11 and LEC11A mutants arose by a cis-dominant mechanism probably due to rearrangement of a cgFUT6 gene. By contrast, LEC11B cells arose by a trans-recessive mechanism, due to the loss of a negative regulatory factor that controls expression of the cgFUT6B gene.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes and buffers were from Boehringer Mannheim, New England Biolabs (Beverly, MA), Promega (Madison, WI), and Life Technologies, Inc. T4 DNA ligase, alkaline phosphatase, proteinase K, and DNase I were from Boehringer Mannheim. T4 polynucleotide kinase, RQ1 DNase, RNasin, Klenow fragment, rATP, rCTP, rGTP, and rUTP were from Promega. Superscript II reverse transcriptase, terminal deoxynucleotidyltransferase, RNase H, DNA molecular weight markers, G418, fetal bovine serum, bovine calf serum, fetal bovine albumin, G418, and rATP, rCTP, rGTP, and rUTP were from Promega. Supernatant II reverse transcriptase, terminal deoxynucleotidyltransferase, RNase H, DNA molecular weight markers, G418, fetal bovine serum, bovine calf serum, α-medium, Opti-MEM I Reduced Serum Medium were from Life Technologies, Inc. 51Cr, [α-32P]dCTP, [γ-32P]ATP, and GDP-[32P]fucose (200 mCi/mmol) were from NEN Life Science Products. The deoxyribonucleotide triphosphates (dNTPs) were from Perkin-Elmer or Boehringer Mannheim. Trypsin was from NEN Life Science Products. The deoxyribonucleotide triphosphates (dNTPs) were from Perkin-Elmer or Boehringer Mannheim. Synthetic oligonucleotides were from the DNA Synthesis Facility of Albert Einstein College of Medicine. Hybrid Nylon membrane and Rapid-hyb buffer were from Amersham Pharmacia Biotech. Nonidet P-40, dimethyl sulfoxide, MOPS, PIPEs, polyethylene glycol, N-acetyllactosamine (Gal[b1,4]GlcNAc; LacNAc), T type 1 acceptor (Gal[b1,3]GlcNAc), 2- fucosyllactose, fetuin, sodium cacodylate, N-ethylmaleimide (NEM), and 2,3-dehydro-2-deoxy-N-acetylenuridine acid were from Sigma. Sialic acid (2,3)GalNAc and unlabeled GDP-fucose were from Oxford Glycosystems (Weybridge, MA). Fuc01,2[Gal[b1,3]GlcNAc-R where R is O(\text{CHO})_{2}O-Me was a kind gift of Dr. Stefan Oscarsson (Stockholm University, Sweden) and LacNAc(Pro) and 2- fucosyl-LacNAc were the kind gifts of Dr. Kushi Matta (Roswell Park, Buffalo, NY). N-Methyl-N-nitrosoguanidine was from ICN Biomedicals, Costa Mesa, CA, and ethylenesulfonate was from Eastman Kodak Co. Sheep red blood cells were obtained from P.M.L. Microbiologicals (Richmond, British Columbia, Canada). Dowex 1 x 4 (100–200 mesh) chloride form was from Bio-Rad. Other chemicals and reagents were from either Sigma or Fisher. The monoclonal antibody anti-SSEA-1 was prepared by 40% ammonium sulfate precipitation of ascites produced by CAF1 mice injected with the hybridoma cell line 480 obtained from Dr. Barbara Knowles (Jackson Laboratories, Bar Harbor, ME). The purified CSLEX-1 monoclonal antibody was obtained from Dr. Paul Terasaki (University of California, Los Angeles, CA), and the VIM-2 monoclonal antibody was the generous gift of Dr. Bruce Maher (San Francisco State University, San Francisco). Anti-selectin monoclonal antibody H18/7, IgG2a, was provided by Dr. Michael Bevilaqua. Rabbit anti-mouse IgM was from Zymed Laboratories Inc. (San Francisco) and 123I-protein A (1000 cpm/mg protein) was from Amersham Pharmacia Biotech. Recombinant human Fuc-TV and human Fuc-TV were from Calbiochem. Interleukin-1β was from Genetics Institute (Cambridge, MA). Lectins including wheat germ agglutinin (WGA), agglutinins from Phaseolus vulgaris (E-PHA and L-PHA), and ricin were from Vector Laboratories, Burlingame, CA. Eculom was from ICN Biomedicals (Costa Mesa, CA). Chinese hamster liver was provided by Dr. Peter Weksnora (University of Wisconsin, Madison, WI).

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Membranes were finally washed in 1×3×trophoresis in 5 or 8% polyacrylamide gels containing 8M urea at 50–80 min. Digestion products were denatured and separated by electrophoresis (from CHO or hybrid cells was prepared using 1 ml of TRIzol Reagent using various restriction enzymes from Boehringer Mannheim. DNA kit (Qiagen). Digestion of genomic DNA to completion was accomplished using standard proteinase K method or the Blood & Cell Culture DNA maxi package version 9.1, Geneworks version 2.5, CLUSTAL W version 1.7 (31), and SCANPS version 2.3.1 (32).

Transfection of the Cloned FUT Gene—Purified plasmid DNA (2 μg) was mixed with sPl2vnea DNA (2 μg) and transfected into Pro 5 CHO cells using the Polybrene method described previously (33). Transfectants selected for resistance to G418 (1.0 mg/ml active weight) were screened for the expression of α,1,3Fuc-T activity by their ability to bind an α,1,3/1,4-siaga plasmas.

Northern Blot Analysis and Ribonuclease Protection—Total RNA from CHO or hybrid cells was prepared using 1 ml of TRIzol Reagent (Life Technologies, Inc.) for 105 cells to obtain 150 μg of RNA. Total RNA, 15 μg of antisense RNA, and 15 μg of sense RNA, using the antisense primer given in the respective figure legend. After reverse transcription, 1 μl of RNA Nase H (2.5 units/μl) was added, and the RNA template was digested by incubation at 55 °C for 20 min. The reaction (100 μl) was extracted once with phenol:chloroform:IAA (25:24:1), and cDNA products were purified through a Sephadex G-50 Quick Spin Column (Boehringer Mannheim). To add poly(A), 15 μl of cDNA was heated at 95 °C for 2 min and quickly chilled on ice before adding 2 μl of 5× terminal deoxynucleotidyltransferase buffer (Life Technologies, Inc.), 2 μl of dATP (2.5 mM) and 1.5 μl of terminal deoxynucleotidyltransferase (18 units/μl). After incubation at 37 °C for 15 min, the reaction was heated to 70 °C for 15 min, diluted to 100 μl, and passed through a G-50 column. For second round PCR amplification, 10 μl of cDNA product was used with oligo(dT)/anchor primer GATCAGAATTCAGCGGCCGCACC(T)19 and the relevant gene-specific nested oligonucleotides and subsequently with primers derived from the known sequence of the FUT gene. DNA and protein sequence analyses were performed using the GCG Sequence Analysis Software Wisconsin Package version 9.1, Geneworks version 2.5, CLUSTAL W version 1.7 (31), and SCANPS version 2.3.1 (32).

Reverse Transcription (RT) and Polymerease Chain Reaction (PCR)—For reverse transcription, 1–2 μg of poly(A)+ RNA, 15 pmol of antisense primer, and 1 unit/μl RNasin were heated to 75 °C for 10–15 min and rapidly chilled on ice to room temperature before further processing. These two fragments were separated by 0.8% agarose-gel electrophoresis at 40 V overnight followed by transfer to Hybond nylon membrane (Amersham Pharmacia Biotech) with 20× SSC. After UV cross-linking in a Stratamax linker (Stratagene), the membranes were hybridized in Rapid-Hyb buffer at 65 °C for 3 h. DNA fragments were labeled with [α-32P]dCTP using PrimeIT RmT kit (Stratagene) to a specific activity of 109 cpm/μg DNA with dCTP, DNA was hybridized, blots were finally washed at 60 °C in 0.2× SSC containing 0.2% SDS for 30 min and exposed at ~70 °C to Kodak X-Omat films with intensifying screens. After rehybridization, blots were erased by boiling in 0.1% SDS.

Southern Blot Analysis—Genomic DNA was prepared by either a standard proteinase K method or the Blood & Cell Culture DNA maxi kit (Qiagen). Digestion of genomic DNA to completion was accomplished using various restriction enzymes from Boehringer Mannheim. DNA fragments were separated by 0.8% agarose-gel electrophoresis at 40 V overnight followed by transfer to Hybond nylon membrane (Amersham Pharmacia Biotech) with 20× SSC. After UV cross-linking in a Stratamax linker (Stratagene), the membranes were hybridized in Rapid-Hyb buffer at 65 °C for 3 h. DNA fragments were labeled with [α-32P]dCTP using PrimeIT RmT kit (Stratagene) to a specific activity of 109 cpm/μg DNA with dCTP, DNA was hybridized, blots were finally washed at 60 °C in 0.2× SSC containing 0.2% SDS for 30 min and exposed at ~70 °C to Kodak X-Omat films with intensifying screens. After rehybridization, blots were erased by boiling in 0.1% SDS.

Reverse Transcription (RT) and Polymerease Chain Reaction (PCR)—For reverse transcription, 1–2 μg of poly(A)+ RNA, 15 pmol of antisense primer, and 1 unit/μl RNasin were heated to 75 °C for 10–15 min and rapidly chilled on ice to room temperature before further processing. These two fragments were separated by 0.8% agarose-gel electrophoresis at 40 V overnight followed by transfer to Hybond nylon membrane (Amersham Pharmacia Biotech) with 20× SSC. After UV cross-linking in a Stratamax linker (Stratagene), the membranes were hybridized in Rapid-Hyb buffer at 65 °C for 3 h. DNA fragments were labeled with [α-32P]dCTP using PrimeIT RmT kit (Stratagene) to a specific activity of 109 cpm/μg DNA with dCTP, DNA was hybridized, blots were finally washed at 60 °C in 0.2× SSC containing 0.2% SDS for 30 min and exposed at ~70 °C to Kodak X-Omat films with intensifying screens. After rehybridization, blots were erased by boiling in 0.1% SDS.
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Results

Selection of Independent Gain-of-Function Mutants with a LEC11 Phenotype—Independent LEC11 mutants were obtained by selecting for resistance to WGA and screening surviving colonies for expression of LeX using the anti-LeX monoclonal antibody α-SSEA-1 conjugated to sheep red blood cells. LEC11A and LEC11B mutants were isolated from two separately mutagenized populations. They have lectin resistance properties similar to LEC11 cells (23) being 2–4-fold resistant to L-PHA, E-PHA, and WGA and ~5–10-fold hypersensitive to ricin and abrin compared with parental CHO cells. Both new mutants expressed the fucosylated determinants LeX, sialyl-LeX, and VIM-2 at similar levels to LEC11 cells (Fig. 1A) and, like LEC11 cells, bound E-selectin expressed on activated human umbilical vein endothelial cells that had been treated with interleukin-1β (IL-1β) in the presence or absence of an antibody to E-selectin (H18/7), and binding was determined as described under “Experimental Procedures.”

Each LEC11 mutant expresses the cloned FUT gene.

Isolation of a CHO FUT Gene That Is Expressed in LEC11, LEC11A, and LEC11B Cells—A FIXII genomic DNA library prepared from Lec1 CHO cells (58) was screened with a human FUT3 gene coding region probe to obtain phage A6.1. An ~9-kb EcoRI fragment and a 3.2-kb SacI subclone were co-transfected with pSV2neo into parental Pro5 CHO cells that lack endogenous α(1,3)Fuc-T activity. Both clones gave more than 30% G418-resistant transfectants which bound αSSEA-1/εSBC, whereas pSV2neo transfectants were uniformly negative. The encoded CHO α(1,3)Fuc-T activity was inhibited by NEM and had the same acceptor specificities as the LEC11 α(1,3)Fuc-T (data not shown).

The 3.2-kb SacI fragment contained a long open reading frame homologous to the coding region of the human FUT5, FUT3, and FUT6 genes (73, 72.1, and 71.9% identical respectively) but considerably different from the FUT4 (50.8% identical), FUT7 (53.2% identical), and the Fuc-TIX cDNA (50.5% identical) coding sequences. Northern analysis with coding region probe 2 gave a hybridization signal of ~1.8 kb with RNA from each LEC11 mutant but not with RNA from parental CHO (Fig. 2A). Ribonuclease protection with riboprobes transcribed from probes 2 and 3, respectively, showed that each LEC11 mutant expresses the same or a highly homologous FUT gene. The 5′-riboprobe of 668 nt protected a sequence spanning nt 12–617 (Fig. 2B) and the 3′-riboprobe of 415 nt protected a sequence spanning nt 638–1053 of the FUT gene coding region (Fig. 2C). No transcripts were protected in parental CHO cell poly(A)+ RNA. In addition, other gain-of-function CHO mutants that possess a biochemically distinct 1,3Fuc-T such as LEC12 (15–17) and LEC30 (24) also did not express this CHO FUT gene.

The Cloned CHO FUT Gene Is Orthologous to Human FUT6—The coding region of the cloned FUT gene contains an ATG that conforms to the Kozak consensus sequence (37) and predicts a polypeptide of 362 amino acids (Fig. 3A). Hydropathy analysis (38) revealed a single hydrophobic membrane spanning domain of 20 amino acids near the N terminus, which
A

![Sequence of the cloned CHO FUT gene.](image)

B

![CLUSTAL W analysis of the CHO FUT coding amino acids](image)

**FIG. 3. Sequence of the cloned CHO FUT gene.** A, the nucleotide sequence and predicted amino acid sequence of the CHO FUT gene cloned from Lec1 genomic DNA are shown. The putative transmembrane domain identified by Kyte Doolittle hydropathy analysis (38) is underlined. Conserved Cys residues typical of NEM-sensitive fucosyltransferases are boxed. Potential N-linked glycosylation sites are shaded.

This sequence has been deposited in GenBank™ data bank (accession number U78737). B, CLUSTAL W analysis of the CHO FUT coding amino acids compared with human Fuc-TVI (accession number L01698). Identical residues are black and similar residues are shaded. The position of amino acids postulated to confer Fuc-TVI acceptor specificity in the human enzyme are starred. The CHO Fuc-T is most similar to human Fuc-TVI.
Each LEC11 α(1,3)Fuc-T is most similar to human Fuc-TVI.

Extracts from LEC11, LEC11A, and LEC11B cells and recombinant hFuc-TV and hFuc-TVI (0.1–0.125 milligrams added to CHO extract so that the total protein concentration was similar) were assayed with the acceptors shown at a concentration of 0.1 μmol and with 4 nmol [3H]GDP-fucose (32,000 cpm) per reaction. Data are the average of duplicate reactions that differed by <5%. Specific activities (pmol/min/mg protein) that correspond to 100% were as follows: LEC11B, 23; LEC11A, 44; LEC11, 24; hFuc-TV, 49; hFuc-TVI, 144.

| Acceptor | α(1,3)Fuc-T activity % |
|----------|------------------------|
| Galβ1,4GlcNAc | 100 100 100 100 100 |
| SAα(2,3) Galβ1,4GlcNAc | 302 228 112 178 153 |
| Fucα(1,2) Galβ1,3GlcNAc | 443 3 <1 2 ≤5 |
| Fucα(1,2) Galβ1,4Glc | 188 10 <1 <1 <1 |

Table I

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Table II

| PROBES | A | H | K |
|--------|---|---|---|
| PROBE 1 | CHL | E | H |
| PROBE 2 | EcoRI | KpnI | HindIII |

Fig. 4. Rearrangements of a cgFUT6 gene in genomic DNA from LEC11 and LEC11A cells. The diagram shows a partial restriction map of the cloned cgFUT6 gene (X: XbaI; A: AvaI; H: HindIII; K: KpnI) with the coding region shaded. For Southern analysis, 15 μg of genomic DNA was digested with the restriction enzymes shown, electrophoresed, transferred to membrane, and probed with locus-specific probe 1 or coding region probe 2. A, membrane on the left was hybridized with probe 2, erased, and rehybridized with probe 1 (right). CHL, Chinese hamster liver DNA; E, EcoRI; H, HindIII; B, DNA digested with BamHI and hybridized to probe 2. C, DNA digested with AvaI and hybridized to probe 2.

data not shown). Since these enzymes do not cut within the sequence of coding region probe 2, the data suggest that the Chinese hamster (cg) genome contains two, highly homologous cgFUT 6 genes and that their integrity and location is maintained in the genome of CHO cells.

Southern analyses with coding region probe 2 identified an additional band in genomic DNA from both LEC11 and LEC11A cells that was absent from Chinese hamster liver, CHO, and LEC11B DNA (Fig. 4, A–C). This extra hybridizing fragment was of weaker intensity indicating that it came from one copy of one allele of a cgFUT6 gene. Five of six restriction enzymes examined detected an extra fragment in LEC11A DNA (EcoRI, 5.2 kb; HindIII, 3 kb; BamHI, 6.3 kb; BglII, 9.7 kb; KpnI, 6.5 kb) and three detected an extra fragment in LEC11 DNA (BamHI 7.8 kb; KpnI, 7.2 kb; AvaI, 5 kb). Therefore, one copy of a CHO cgFUT6 gene in both LEC11A and LEC11 cells appears to have undergone rearrangement leading to expression of the gene. By comparing Southern patterns obtained with probes 1 and 2, it can be deduced that the breakpoint for the rearrangement in LEC11A DNA occurred in the −0.5-kb region between probes 1 and 2, just upstream of the cloned CHO cgFUT6 gene-coding region. This type of cis rearrangement in LEC11 and LEC11A cells is not predicted to be relevant to the in vivo regulation of a FUT6 gene. However, no rearrangements were detected in genomic DNA from LEC11B cells. Therefore, the mechanism of activation of a cgFUT6 gene in this mutant was examined by somatic cell hybrid analysis.
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A trans-Acting, Negative Regulatory Factor That Controls cgFUT6 Gene Expression Is Inactive in LEC11B Cells—In order to determine whether a cis- or trans-genetic mechanism caused expression of a cgFUT6 gene in LEC11B cells, somatic cell hybrids were assayed for the ratio of cgFUT6 gene transcripts with actin transcripts. In a cis mechanism of gene activation, a rearranged cgFUT6 allele would continue to be transcribed in hybrids formed with CHO cells, and the cgFUT6 genes in the CHO genome would remain silent, giving a cgFUT6:actin transcript ratio close to 0.5. In a trans-positive mechanism, a gene other than a cgFUT6 gene would be affected, and its product would activate a cgFUT6 gene in the mutant and in the parent CHO genomes. In this case, the cgFUT6:actin transcript ratio would be close to 1.0. In a trans-negative mechanism of gene activation, the negative regulator that keeps cgFUT6 genes silent in CHO cells would suppress the cgFUT6 gene expressed in the mutant, and the cgFUT6:actin transcript ratio would be close to zero.

Independent hybrids between CHO parental cells and LEC11, LEC11A, or LEC11B cells were isolated, and total RNA was subjected to Northern analysis using coding region probe 2 followed by an actin gene probe. The results in Fig. 5A show that LEC11 × CHO and LEC11A × CHO hybrids contained readily detectable cgFUT6 gene transcripts, as predicted if they arose by a cis mechanism. However, LEC11B × CHO hybrids had little or no hybridizing signal with probe 2, despite equivalent hybridization to the actin probe. The suppression of cgFUT6 gene transcripts in these hybrids provides evidence for the action of a negative regulatory factor encoded by the CHO genome. Consistent with this, hybrids formed between LEC11B and an unrelated glycosylation mutant LEC18 that should also contain the negative regulator had zero or low levels of cgFUT6 gene transcripts by Northern analysis (Fig. 5B). The presence of low levels of cgFUT6 gene transcripts in hybrids formed with LEC11B cells may be due to limiting amounts of the trans-acting negative regulator. Alternatively, the gene encoding the negative regulator could be lost in a few hybrids due to chromosomal segregation. Although CHO cell hybrids are relatively stable, chromosomal segregation occurs at frequencies of ~10⁻³ per cell per generation (45).

The ratio of cgFUT6:actin gene transcripts was calculated by densitometry of Northern signals (Table II). In all hybrid combinations that included LEC11B, the cgFUT6:actin transcript ratio was the level predicted for a trans-negative mechanism: in LEC11B × CHO hybrids the transcript ratio was ≤0.1; in hybrids formed with either LEC11 or LEC11A cells the transcript ratio was ~0.5.

Additional evidence for the presence of a negative regulator of cgFUT6 gene expression in CHO cells that is inactive in LEC11B cells was obtained by α(1,3)Fuc-T assays of hybrid extracts. CHO × LEC11B hybrids had low levels of α(1,3)Fuc-T activity, much lower than predicted from the combined α(1,3)Fuc-T activity of LEC11B and parent cells (Table III). This negative effect was not due to the presence of an inhibitor of α(1,3)Fuc-T activity in CHO cells, since extract mixing experiments gave additive results. This recessive behavior of the LEC11B phenotype was also observed in lectin resistance tests. LEC11B × CHO hybrids were not resistant to WGA or hyper-sensitive to ricin. The combined data provide strong evidence that the cgFUT6 gene is expressed in LEC11B cells due to an inactivating mutation in a gene that encodes a negative regulatory factor.

**Table II**

| Cells fused       | No hybrids tested² | cgFUT6:actin transcripts observed² | Ratio predicted for cis mechanism² |
|-------------------|--------------------|------------------------------------|-----------------------------------|
| LEC11 × CHO       | 5                  | 0.65 ± 0.24 (n = 21)               | 0.5                               |
| LEC11A × CHO      | 4                  | 0.29 ± 0.13 (n = 10)               | 0.5                               |
| LEC11B × CHO      | 5                  | 0.10 ± 0.04 (n = 12)               | 0                                  |
| LEC11B × LEC11A   | 5                  | 0.44 ± 0.12 (n = 10)               | 0.5                               |
| LEC11B × LEC11    | 5                  | 0.60 ± 0.29 (n = 12)               | 0.5                               |

*² Number of independent hybrids tested.

*² Average and standard deviation of densitometry results of cgFUT6 gene transcripts:actin gene transcripts. n = number scanned.

*² Predicted value of densitometry result of each hybrid for a cis- or trans-mechanism of cgFUT6 gene expression.

**Table III**

| Cell line     | Exp. | α(1,3)Fuc-T activity (10⁻⁶ pmol/min/mg protein) | Observed² | Predicted³ |
|---------------|------|-----------------------------------------------|-----------|------------|
| CHO           | 1    | 0.6                                           |           |            |
|               | 2    | <2.1                                          |           |            |
| LEC11B        | 1    | 115.3                                         |           |            |
|               | 2    | 229.3                                         |           |            |
| LEC11B × CHO  | 1    | 20.0                                          | 115.9     |            |
|               | 2    | <2.2                                          | 231.4     |            |
|               | 2    | 12.7                                          | 231.4     |            |
|               | 2    | 67.7                                          | 231.4     |            |

*² Observed α(1,3)Fuc-T activity from cell lysates.

*³ Expected α(1,3)Fuc-T activity for each cell hybrid, assuming activities are additive.

*³ In experiment 2, three independent hybrids were assayed.

The substrate used in the α(1,3)Fuc-T assays was Galβ1,4GlCNac (LacNAc).
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LEC11B transcripts have divergent 3'- and 5'-UTR sequences. A, 3'-RACE of poly(A)+ RNA from LEC11, LEC11A, and LEC11B cells using the oligo(dT)/anchor primer (“Experimental Procedures”) for reverse transcription. First round cDNA products were amplified with the anchor primer ("Experimental Procedures") and primer 152 (AGGCCTCCGGTCTAGTACGG; sense) from the 3′ end of the cgFUT6 gene coding sequence (Fig. 3). cDNA products were cloned, and ≥2 independent clones were sequenced from both strands. LEC11 and LEC11B sequences were identical and differed from LEC11A. The arrow marks the beginning of divergent sequence and TGA stop codon is double underlined, and the poly(A)+ addition sequence is boxed. B, the 3'-UTR obtained from LEC11A poly(A)+ RNA is aligned with the corresponding sequence of the cloned cgFUT6 gene (Fig. 3). The TGA stop codon is double underlined, and the putative poly(A)+ addition sequence is underlined. C, for 5'-RACE, poly(A)+ RNA from LEC11B cells was subjected to RT-PCR using primer 122 (TGCGCTGGAGCCTCGAG; antisense) near the 5′ end of the cgFUT6 gene coding region (Fig. 3). After addition of poly(A) by terminal deoxynucleotidyltransferase, cDNA products were amplified using the oligo(dT)anchor primer and primer 121 (CATCTCTCCAGTACGATGACG; antisense). Two 5'-RACE sequences are aligned with the sequence upstream of the cloned cgFUT6 gene-coding region (Fig. 3). The ATG initiation codon is double underlined. D, the coding region of the cgFUT6 gene expressed in LEC11B cells was obtained by RT-PCR from poly(A)+ RNA using primers from the 5'- and 3'-UTR sequences that span the coding region. The RT reaction was performed with 3′-UTR antisense primer A22 (GAGCCACCTACTGAGATGCTCC); PCR of cDNA products was performed with the 5′-UTR sense primer A21 (CGCTCAGTCTCAGGAGCAG; sense) from genomic DNA of Gat+2 parental CHO cells using primer 257B (GATC-CCCCCAGGCGATGATT; sense) immediately upstream of the cgFUT6B coding region and primer 171 (AGCTCATTCTTCTAGTACGATGTCC; antisense) from the 3′-UTR region. Genomic DNA from Pro 5 and Gat+2 parental CHO cells was used to obtain the sequence of the cgFUT6A gene with primer 257A (GGACTACCAAGGCATGATT; sense) immediately upstream of the coding region and primer 169 (CTGACAGATAGTCTACGATG; antisense) from the unique 3'-UTR region. This sequence differed by 2 nucleotides (G214C and G225C numbered from the ATG). A probe derived from this sequence hybridized only to cgFUT6B gene transcripts in LEC11B cells (Fig. 7). This 5′ exon was shown to be linked to the coding region by sequencing of RT-PCR products from primers that spanned the 5′-UTR and coding exons (data not shown). The fact that the 5′ exon was not present in LEC11 or LEC11A cgFUT6 gene transcripts provides further evidence for rearrangement of the respective cgFUT6 genes transcribed in these mutants.

The transcript-specific 3'UTR probes and coding region probe 2 were used in Southern analyses to identify hybridizing fragments corresponding to the distinct cgFUT6 genes (Fig. 8). The 3'UTR probe that hybridized solely to transcripts from LEC11A cells hybridized to only one of the two DNA fragments detected by coding region probe 2. Therefore, this fragment contains the cloned cgFUT6 gene that is functionally expressed only in LEC11A cells and is henceforth termed cgFUT6A. The 3′-UTR probe that hybridized solely to transcripts from LEC11 and LEC11B cells was found by Southern analysis to hybridize to the second genomic DNA fragment identified by coding region probe 2. Therefore this probe hybridized to the second functional cgFUT6 gene, henceforth termed cgFUT6B.

Additional evidence for the existence of two FUT6 genes was obtained by sequencing PCR products derived from genomic DNA of Pro 5 and Gat+2 parental CHO cells and RT-PCR products from LEC11B transcripts. Gene-specific primers were designed from sequences immediately upstream of the respective cgFUT6A and cgFUT6B coding regions and paired with primers from the unique 3′-UTR region of each gene (see Fig. 6A). With cgFUT6A gene-specific primers, CHO genomic DNA gave coding region sequence that was identical to the cloned gene from Lec1 cells in Fig. 3, except for two nucleotide differences (G214C and G225C numbered from the ATG). The latter
presumably reflects cgFUT6A gene mutations present in the Lec1 genome or that arose during cloning. These changes translate into two amino acid differences (A72P and R75S; Fig. 6D). With cgFUT6B gene-specific primers, CHO genomic DNA gave a coding region sequence identical to that obtained by RT-PCR with primers from the 5'-UTR and 3'-UTR sequences unique to LEC11B transcripts. The coding region sequence of the cgFUT6B gene differed from the cgFUT6A gene sequence in seven nucleotides that translated into only two amino acid differences (Fig. 6D).

cgFUT6B Gene Transcripts Are Suppressed in LEC11A × LEC11B Hybrids— If a negative regulatory factor represses expression of the cgFUT6B gene in LEC11B cells by a trans mechanism, and the cgFUT6A gene is expressed in LEC11A cells by a cis mechanism, hybrids formed between LEC11A and LEC11B cells should suppress expression of cgFUT6B gene transcripts and express mainly cgFUT6A gene transcripts. The Northern blots in Fig. 9 show this to be the case. Independent LEC11A × LEC11B hybrids contained predominantly cgFUT6A gene transcripts. Densitometry analyses of the FUT6:actin signal ratio for hybrids with the cgFUT6A gene-specific probe averaged 0.88 compared with 0.94 for LEC11A RNA and 0.02 for LEC11B RNA (Fig. 9A). By contrast, the ratio for the cgFUT6B probe averaged 0.1 for hybrid transcripts (Fig. 9B). A 4-fold difference was obtained between LEC11A-specific and LEC11B-specific transcripts in hybrids, similar to the ratio observed in LEC11B × CHO and LEC11B × LEC18 hybrids (Fig. 4 and Table II). Therefore, LEC11A × LEC11B hybrids expressed predominantly transcripts from the cgFUT6A gene which is functional in LEC11A cells, whereas transcripts from the cgFUT6B gene were suppressed. Consistent with this, fucosyltransferase activities of two LEC11A × LEC11B hybrids were those expected if only one cgFUT6A gene was active. Thus, the trans-negative regulatory factor contributed by the LEC11A genome specifically suppressed expression of the cgFUT6B gene in both the LEC11A and the LEC11B genome.

DISCUSSION

Gain-of-function CHO mutants have provided access to several developmentally regulated glycosyltransferase activities, including two novel GlcNAc-T activities that generate new N-glycan cores not available from any other source (14, 46). In this paper, characterization of three gain-of-function LEC11 mutants has shown that two of them arose due to a cis mechanism of gene rearrangement that resulted in expression of one of two cgFUT6 genes, whereas the third arose due to the loss of
an NRF that suppresses expression of only the cgFUT6B gene in CHO cells (Table IV). Neither cgFUT6 gene gave detectable transcripts in poly(A)^+ RNA from CHO cells. The cgFUT6 genes therefore appear to be transcriptionally silent in CHO cells, since even very short-lived messenger RNAs such as myc gene transcripts with a half-life of only 9 min are detected by Northern analysis (47).

While the LEC11 and LEC11A mutants provide a source of two α(1,3)Fuc-TVI enzymes and may be useful for studies of selectin cell adhesion mechanisms, they presumably do not provide insights into molecular mechanisms that regulate FUT6 gene expression in vivo. The gene rearrangements detected by Southern analysis probably arose during culture or due to mutagenesis. By contrast, the negative regulatory factor (NRF) inactivated by the LEC11B mutation is likely to function in vivo. Northern and α(1,3)Fuc-T analyses of hybrids formed with LEC11B and several other cell types clearly show that CHO cells and CHO glycosylation mutants with other mutations, including LEC11A cells, encode the NRF that suppresses expression of the cgFUT6B gene. Experiments are in progress to isolate this factor. It is clearly not a molecule that recognizes a sequence in either the coding or 3'-UTR regions of the cgFUT6B gene because LEC111 cells, which carry the NRF, express stable cgFUT6B gene transcripts (Fig. 7). It could be an NRF that binds to the 5'-UTR sequence unique to cgFUT6B transcripts in LEC11B cells or a splicing factor that normally splices out transcripts of the cgFUT6B gene. However, it seems most likely to be a negative regulator of transcription of the cgFUT6B gene that acts on a negative regulatory element (NRE) in the promoter region of this gene. Although there have been no reports of endogenous positive or negative transcriptional factors that control expression of the FUT6 genes that encode an α(1,3)Fuc-T, there is precedence for this form of transcriptional control in at least two glycosyltransferase genes. The β(1,4)Gal-T1 gene is differentially regulated during lactation by an NRF that binds to an NRE located a few nucleotides upstream of the ATG (48) and the dolichol-P-GlcnAc-T gene that is regulated during mammary gland development has an NRE located −1 kb upstream of the ATG (49, 50).

The FUT6 gene encodes the major α(1,3)Fuc-T expressed in human liver (11), and analysis of humans with no Fuc-TVI activity have shown that Fuc-TVI is responsible for fucosylating glycoproteins that are secreted from liver (51). Humans with a point mutation that inactivates α(1,3)Fuc-TVI activity appear to suffer no ill effects (51, 52), but it remains to be seen whether they exhibit a differential sensitivity to microbial pathogens or liver toxins. The NRF we have found in CHO cells is predicted to be responsible, at least in part, for keeping the FUT6 gene silent in the tissues where it is not expressed, such as lung (11). In human pancreas the FUT6 gene is transcriptionally silent, but it is expressed in pancreatic tumors (53). This may reflect down-regulation of a FUT6 gene NRF, leading to enhanced expression. This may in turn lead to metastasis following expression of Le^a^ and SLe^a^ on cancer cells. The NRF that regulates FUT6B expression in CHO cells may be involved in this type of control. Although the cgFUT6B genes are almost identical in their coding regions, the regulation of their expression is independent because each LEC111 mutant expresses only one of the two genes. Identifying mechanisms that regulate specific fucosylation events is critical to understanding biological roles of the fucose residues transferred by different fucosyltransferases. The reactivation of FUT6 genes silenced during development and differentiation occurs in cancer, and the expression of SLe^a^ is associated with poor prognosis, pos-

**Fig. 7.** Northern analysis using gene-specific probes. Total RNA (10–15 μg) from CHO and LEC11 mutants was electrophoresed, transferred to membrane, and hybridized to probes derived from 3'-RACE and 5'-RACE products or coding region probe 2 (Fig. 2) or the actin probe. The 5'-UTR probe specific for LEC11B transcripts was an EcoRI fragment of 150 bp derived from a cloned 5'-RACE product obtained by RT-PCR from LEC11B poly(A)^+ RNA using primer 143 (TTTGCGAGCCAAAGCTCTACTGC; antisense) based on sequence unique to the LEC11B 5'-UTR (Fig. 6C). The 3'-UTR probe (275 bp) specific for transcripts of LEC11A cells was obtained by PCR of a cloned cDNA obtained by 3'-RACE of poly(A)^+ RNA from LEC11A cells using sense primer 168 (GTGCTAGACACTCCCTTGATGAGC) and antisense primer 169 (CTGACAGAATAAGGTCTCATCTGG). The 3'-UTR probe (324 bp) specific for transcripts of LEC11B and LEC11C cells was obtained by PCR of a cloned cDNA obtained by 3'-RACE of LEC11B poly(A)^+ RNA using sense primer 170 (TGCCCCTGTGTGGTCGTCTTGCC) and antisense primer 171 (AGCTTACATTTCTAGATCACTCCT).

**Fig. 8.** Southern analysis using gene-specific probes. Gene-specific probes were derived from the 3'-UTR sequence unique to the LEC11A and LEC11B transcripts, respectively, as depicted in the diagram. Sequence differences between the coding regions of each transcript are denoted by stars in LEC11B. Genomic DNA (10–15 μg) from CHO cells and LEC11 mutants was digested with EcoRI, electrophoresed, transferred to membrane, and hybridized to coding region probe 2 (see diagram) or the probe specific for the 3'-UTR region of LEC11C transcripts in order to identify the cgFUT6A gene, or the probe specific for the 3'-UTR of LEC11B transcripts to identify the cgFUT6B gene (see Fig. 7). Slight degradation of LEC11B genomic DNA is apparent in the middle blot. The cgFUT6B gene-specific probe gave background hybridization but hybridized at high stringency only to the 2.3-kb fragment that contains cgFUT6B.
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assays (55, 56). Phylogenetic analysis of the Lewis genes subfamily, lead Costache et al. (55) to propose that duplication events at the origin of the present cluster of human genes (FUT6-FUT3-FUT5) appeared between the great mammalian radiation (80 million year ago) and the separation of human and chimpanzee (10 million years ago). The three Lewis genes are predicted to have arisen from two gene duplications, the most recent of which occurred just before the separation of man and anthropoid apes from the main evolutionary trunk (55). The Lewis precursor gene was proposed to be the bovine gene that is most similar to hFUT6. Although physical linkage of the Chinese hamster FUT6 genes has not been proven, preliminary data from Southern analyses and restriction mapping of cloned genomic DNA fragments are consistent with this probability.2

A phylogenetic tree based on protein distance between the human Lewis enzymes, the bovine Fuc-T, and CHO Fuc-TVIA and Fuc-TVIB sequences was constructed using the PHYLIP Phylogeny Interference Package 3.5c of programs and the Fitch-Margoliash least squares method with an evolutionary clock (57). The tree was drawn from the PHYLIP dendrogram3 with the DRAWGRAM program. It predicts that the first duplication of the original Lewis gene occurred in lower mammals just before the separation of Chinese hamster from the main evolutionary trunk.

Acknowledgments—We are extremely grateful to all those noted in the text who supplied materials. Thanks also to Subha Sundaram for superb technical assistance and to Olga Blumenfeld and Jihua Chen for helpful discussions.

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2 A. Zhang and P. Stanley, unpublished observations.
3 cis Infobiogen available on-line at the following addresses: E-mail: bioinfo@infobiogen.fr and http://www.infobiogen.fr.

Fig. 9. Transcripts of the cgFUT6B gene are suppressed in LEC11A x LEC11B hybrids. Independent hybrids 1–5 were isolated from a fusion between LEC11A and LEC11B cells. Total RNA isolated from the hybrids or from LEC11A or LEC11B cells was electrophoresed, transferred to membrane, and hybridized with gene-specific probes (see Fig. 7) followed by an actin probe. A, probe specific for cgFUT6B transcripts expressed in LEC11B cells (upper panel). B, probe specific for cgFUT6A transcripts expressed in LEC11A cells. The ratio of FUT6: actin signals was determined for each lane by densitometry and is given in the text.

| GOF mutant | cgFUT6 gene | cgFUT6 gene rearrangements | Activation mechanism |
|------------|-------------|---------------------------|---------------------|
| LEC11A     | cgFUT6A     | EcoRI, HindIII, BamHI, BglII, KpnI | Cis                 |
| LEC11B     | cgFUT6B     | KpnI, AplI, BamHI          | Cis                 |
| LEC11B     | cgFUT6B     | None detected             | Trans (loss of NRF) |

sibly because it correlates with an enhanced metastatic ability. Thus it is important to identify regulatory mechanisms that fail during cancer progression.

The fact that there are two functional Chinese hamster FUT6 genes that have almost identical coding regions is of interest in terms of evolutionary relationships of related FUT genes. The human Lewis genes, FUT3, FUT5, and FUT6 share about 90% sequence identity and are organized in a cluster on band 13.3 of the short arm of chromosome 19, suggesting that they were generated by successive gene duplications followed by divergent evolution. The cluster spans approximately 50 kb, with a distance of ~13 kb between the FUT6 and FUT3 genes (54) and of ~25 kb between the FUT3 and FUT5 genes (6). Only one bovine gene corresponds to the human cluster of the Lewis genes, and when transfected into COS-7 cells it gives rise to an α1,3Fuc-T activity with properties similar to human Fuc-TVI (35). By contrast, each of the human Lewis subfamily genes has a homologue identified in chimpanzee. In fact, each corresponding pair of genes from the two species shares more than 98% primary sequence identity. COS-7 cells transfected with chimpanzee and human genes express similar patterns of cell-surface determinants and acceptor specificities in in vitro
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