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The Gain-of-Function Chinese Hamster Ovary Mutant LEC11B Expresses One of Two Chinese Hamster *FUT6* Genes Due to the Loss of a Negative Regulatory Factor*

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Aimin Zhang, Barry Potvin, Ari Zaiman‡, Wei Chen, Ravindra Kumar§, Laurie Phillips¶, and Pamela Stanley‖

From the Department of Cell Biology, Albert Einstein College of Medicine, New York, New York, 10461 and ¶Cytel Corporation, La Jolla, California 92121

The LEC11 Chinese hamster ovary (CHO) gain-of-function mutant expresses an α(1,3)fucosyltransferase (α(1,3)Fuc-T) activity that generates the Le^X, sialyl-Le^X, 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 α(1,3)Fuc-T expression in mammals, mechanisms of *FUT* gene expression were investigated in LEC11 cells and two new, independent mutants, LEC11A and LEC11B. Northern and ribonuclease protection analyses, using probes that span the coding region of a cloned CHO *FUT* gene, detected transcripts in each LEC11 mutant but not in CHO cells or other gain-of-function CHO mutants that express a different α(1,3)Fuc-T activity. Coding region sequence analysis and α(1,3)Fuc-T acceptor specificity comparisons with recombinant human Fuc-TV and Fuc-TVI showed that the cloned *FUT* gene is orthologous to the human *FUT*6 gene. Southern analyses identified two closely related *FUT*6 genes in the Chinese hamster, whose evolutionary relationships are discussed. The blots showed that rearrangements had occurred in LEC11A and LEC11 genomic DNA, consistent with a *cis* mechanism of *FUT6* gene activation in these mutants. By contrast, somatic cell hybrid analyses revealed that LEC11B cells express *FUT*6 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 *FUT*6 gene transcripts from each LEC11 mutant. Northern and Southern analyses with gene-specific probes showed that LEC11A cells express only the cg*FUT*6A gene (where cg is *Cricetulus griseus*), whereas LEC11 and LEC11B cells express only the cg*FUT*6B gene. In LEC11A × LEC11B hybrid cells, the cg*FUT*6A gene was predominantly expressed, as predicted if a *trans*-acting negative regulatory factor functions to suppress cg*FUT*6B gene expression in CHO cells. This factor is predicted to be a cell type-specific regulator of *FUT*6 gene expression in mammals.

α(1,3)Fucosyltransferases (α(1,3)Fuc-T)[1] 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 α(1,3)Fuc-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 *FUT*7 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 α(1,3)Fuc-T activity (reviewed in Refs. 2 and 3). The *FUT*3, *FUT*5, and *FUT*6 (Lewis) genes reside in a cluster on chromosome 19 (5, 6); the *FUT*4 gene is on chromosome 11 (7), and the *FUT*7 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 Le^X and/or sialyl-Le^X determinants. Fuc-TIII generates in addition the Le^a, Le^b, sialyl Le^a, and sialyl-Le^b determinants (9). The human α(1,3)Fuc-T activities are differentially expressed in adult tissues and in cancer (2, 10, 11). The enhanced ability of cancer cells to express sialyl-Le^X 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 α(1,3)Fuc-T levels and activities in a cell type- or tissue-specific fashion. To date it

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U78737, AF090449, and AF090450.

‡ Present address: Dept. of Medicine, University of Maryland, Baltimore, MD 21210.

§ Present address: Genetics Institute, Inc., 87 Cambridge Park Rd., Cambridge, MA 02140.

‖ To whom correspondence should be addressed: Dept. of Cell Biology, Albert Einstein College Medicine, 1300 Morris Park Ave., New York, NY, 10461. Tel.: 718-430-3346; Fax: 718-430-8574; E-mail: stanley@aecom.yu.edu.

[1] The abbreviations used are: α(1,3)Fuc-T, α(1,3)fucosyltransferases; CHO, Chinese hamster ovary; Galβ(1,4)GlcNAc, Type 1 acceptor; LacNAc, *N*-acetyllactosamine; Galβ(1,3)GlcNAc, Type 1 acceptor; NEM, *N*-ethylmaleimide; Fuc-T, fucosyltransferase; *FUT*, fucosyltransferase gene; cg, *Cricetulus griseus* or Chinese hamster; RT, reverse transcriptase; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; L-PHA, leukoagglutinin from *P. vulgaris*; E-PHA, erythroagglutinin from *P. vulgaris*; WGA, wheat germ agglutinin; NRE, negative regulatory element; NRF, negative regulatory factor; MOPS, 3-(*N*-morpholine)propanesulfonic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); kb, kilobase pair(s); UTR, untranslated region; nt, nucleotides; bp, base pair(s).

is known that the 5′-untranslated regions of the *FUT*3, *FUT*5, and *FUT*6 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 Le^X, sialyl-Le^X, and VIM-2 determinants on cell-surface glycans (15–17). The sialyl-Le^X 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 *FUT*6 gene, whereas CHO cells contain no cg*FUT*6 gene transcripts by RNase protection analysis. Invesigations 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 cg*FUT*6 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 cg*FUT*6B 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, Sp6 RNA polymerase, 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, α-medium, Opti-MEM I Reduced Serum Medium were from Life Technologies, Inc. ^{51}Cr, [α-^{32}P]dCTP, [γ-^{32}P]ATP, [α-^{32}P]CTP, and GDP-[^{14}C]fucose (260.3 mCi/mmol) were 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. Hybond Nylon membrane and Rapid-hyb buffer were from Amersham Pharmacia Biotech. Nonidet P-40, dimethyl sulfoxide, MOPS, PIPES, polyethylene glycol, *N*-acetyllactosamine (Galβ(1,4)GlcNAc; LacNAc), Type 1 acceptor (Galβ1,3GlcNAc), 2′-fucosyllactose, fetuin, sodium cacodylate, *N*-ethylmaleimide (NEM), and 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid were from Sigma. Sialic acidα(2,3)LacNAc and unlabeled GDP-fucose were from Oxford Glycosystems (Wakefield, MA). Fucα(1,2)Galβ-(1,3)GlcNAc-R where R is $O(CH_2)_8O_2Me$ was a kind gift of Dr. Stefan Oscarsson (Stockholm University, Sweden) and LacNAcβbenzyl 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 ethylmethanesulfonate was from Eastman Kodak Co. Sheep red blood cells were obtained from P.M.L. Microbiologicals (Richmond, British Columbia, Canada). Dowex 1 × 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 Caf_1/J 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 Macher (San Francisco State University, San Francisco). Anti-E-selectin monoclonal antibody H18/7, IgG2a, was provided by Dr. Michael Bevilaqua. Rabbit anti-mouse IgM was from Zymed Laboratories Inc. (San Francisco) and ^{125}I-protein A (~10^8 cpm/mg protein) was from Amersham Pharmacia Biotech. Recombinant human Fuc-TV and human Fuc-TVI were from Calbiochem. Interleukin-1β was from Genetics Institue (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. Ecolume was from ICN Biomedicals (Costa Mesa, CA). Chinese hamster liver was provided by Dr. Peter Wejksnora (University of Wisconsin, Madison, WI).

Cell Lines and Cell Culture—CHO parental cell lines, Pro^-5 and Gat^-2, were isolated previously (22). The origin of the CHO mutants LEC11 (Pro^-LEC11.E7) and LEC12 (Pro-LEC12.1B) is described (23), and the LEC30 CHO mutant was isolated as described (24). HL-60 cells were from the American Type Culture Collection (Rockville, MD). All cells were maintained in suspension culture at 37 °C in complete α medium supplemented with 10% fetal bovine serum or 10% bovine calf serum and 1% fetal bovine serum.

Selection of α(1,3)Fuc-T Expressing CHO Mutants—The two new CHO mutants, LEC11A (Pro^-LEC11.E2) and LEC11B(Gat^-LEC11.F2), were isolated form Pro^-5 or Gat^-2 cells following mutagenesis with *N*-methyl-*N*-nitrosoguanidine or ethylmethanesulfonate, respectively, as described (25). Selection was from ~10^7 cells with 3.5 μg/ml (LEC11A) or 7.5 μg/ml (LEC11B) wheat germ agglutinin (WGA) followed by screening of surviving colonies for the expression of cell surface Le^X, using the anti-SSEA-1 monoclonal antibody conjugated to sheep red blood cells, as described (24, 26). Red colonies were picked, expanded, and cloned by limiting dilution. Lectin resistance was determined by titration of cytotoxic lectins in 96-well microtiter dishes as described (27).

Generation of Somatic Cell Hybrids—To obtain somatic cell hybrids, CHO cells carrying the Pro^- auxotrophic marker and CHO cells carrying the Gat^- auxotrophic marker were mixed; fusion was induced by treatment with polyethylene glycol 1000, and dimethyl sulfoxide and hybrids were selected in α-medium lacking glycine, adenosine, thymine, and proline and containing 10% dialyzed fetal calf serum as described (27). Hybrids arose at a frequency of ~10^{-3}. Spontaneous hybrids and revertants arose at frequencies of <10^{-5}. Hybrids were shown by karyotype analysis to be pseudotetraploid as described previously (27).

Monoclonal Antibody Binding—The ability of CHO cells and the three LEC11 cell lines to bind to anti-SSEA-1, CSLEX-1, or VIM-2 monoclonal antibodies was quantitated as described (17, 24). Briefly, 10^6 washed cells were incubated with ~1 μg of antibody in 200 μl of phosphate-buffered saline, pH 7.2, containing 2% bovine serum albumin for 1 h at 4 °C. After washing to remove primary antibody, rabbit anti-mouse IgM antibody was incubated under the same conditions for 1 h. After removal of unbound secondary antibody, ^{125}I-protein A-Sepharose (~100,000 cpm) was added. Following 1 h at 4 °C, bound ^{125}I was counted in a gamma scintillation counter.

E-Selectin Binding Assays—Confluent 48-well cultures of human umbilical vein endothelial cells were prepared as described (28) and stimulated with interleukin-1β (10 μg/ml) for 4 h to induce expression of E-selectin. HL-60 and LEC11 mutant cells were labeled by incubation with 450 μCi of ^{51}Cr per 3 × 10^6 cells. Labeled cells (2 × 10^5) were incubated with phosphate-buffered saline containing 2 μg/ml anti-E-selectin antibody H18/7 (29) or buffer alone in 400 μl. After 30 min at 15 °C, unbound cells were removed following systematic resuspension with a Pasteur pipette. Adherent cells were lysed in 2% SDS containing 10% glycerin and counted in a gamma counter. Percent counts/min in bound cells compared with input cells was plotted as the mean and S.D. of triplicate assay points.

α(1,3)Fuc-T Assays—The preparation of cell-free extracts and the assay conditions used to measure α(1,3)Fuc-T activity were as described previously (24, 26). 2′-Fucosyllactose and 2′-fucosylLacNAc, LacNAc, and SAα(2,3)LacNAc were used as acceptors. Briefly, each reaction mixture contained 2.5 μmol of MOPS (pH, 7.0), 5 μmol of NaCl, 0.25 μmol of $MnCl_2$, 2–4 nmol of GDP-[^{14}C]fucose (10,000 cpm/nmol), 0.1–1.0 μmol of acceptor, and 5–10 μl of cell extract (~100 μg of protein) in a final volume of 50 μl. After incubation at 37 °C for 30 min, the reaction was stopped by the addition of 1 ml of ice-cold deionized, distilled water. When 2′-fucosyllactose or LacNAc was the substrate, all product was eluted from 1.5-ml ion exchange columns (Dowex 1 × 4, Cl^- form) with 2 ml of deionized, distilled water. However, complete elution of product when 2′-fucosylLacNAc or SAα(2,3)LacNAc was the acceptor required an additional wash with 3 ml of 0.1 M or 0.15 M NaCl. Protein concentrations were measured using the Bio-Rad protein assay reagent under conditions recommended by the manufacturer.

Isolation of a Chinese Hamster FUT Gene—Genomic DNA from Lec1 CHO cells was partially digested with *Mbo*I, and fragments of 9–23 kb were isolated using sodium chloride gradient centrifugation. Size-fractionated DNA fragments were partially filled in with dATP and dGTP and ligated to *Xho*I-digested λFIXII (Stratagene, La Jolla, CA) phage arms that also had been partially filled in with dTTP and dCTP. The ligation product was packaged *in vitro* with packaging extracts from Stratagene and titered on KW251 *Escherichia coli* host cells. Approximately 7×10^5 recombinants were transferred to nitrocellulose filters (Schleicher & Schuell) and hybridized at 42 °C to the 944-bp radiolabeled *Xba*I-*Eco*RI fragment isolated from the insert of plasmid pSi+FTE(α1,3/1,4) kindly supplied by Dr. John Lowe (University Michigan, Ann Arbor, MI). This fragment contains most of the coding sequence of the human Lewis enzyme, α(1,3)Fuc-TIII (9). After hybridization for 48 h in 50% formamide, 5× SSC, 10× Denhart's solution, 0.1% SDS, 0.8% dextran sulfate, and 100 μg/ml denatured herring sperm DNA, filters were rinsed twice for 30 min at room temperature in 1× SSC, 0.4% SDS and then three times for 1 h at 45 °C in the same buffer. Hybridizing plaques were purified through three rounds of replica plating.

Phage DNA from clone A6.1 contained an ~9-kb *Eco*RI fragment and a 3.2-kb *Sac*I fragment that hybridized under stringent conditions (0.2× SSC containing 0.2% SDS at 65 °C) to the 944 bp probe. These two fragments and subclones derived from them were cloned into the pBluescript SK(+) (Stratagene), pGEM3Z vector, or pGEM7Zf(+) vectors (Promega), respectively. Plasmids were amplified in *E. coli* strains XL1-Blue or JM109, and plasmid DNA was purified using Qiagen (Valencia, CA) plasmid preparation kits. DNA sequencing on both strands was performed using automated sequencer models ABI373A and ABI337 (Perkin-Elmer). Reactions were primed with vector-specific 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 (30), 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 pSV2neo 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(1,3)Fuc-T activity by their ability to bind an αSSEA-1/sRBC conjugate as described (24, 26). Positive red colonies were cultured and cell extracts tested for α (1,3)Fuc-T activity.

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 10^7 cells to obtain ~150 μg of total RNA. Poly(A)$^+$ RNA was isolated from ~10^8 washed cells using FastTrack 2.0 Kit mRNA Isolation System (Invitrogen, Carlsbad, CA) to obtain poly(A)+ RNA. RNA was separated on a 1.2% formaldehyde-agarose gel, transferred to Hybond nylon membrane (Amersham Pharmacia Biotech), and hybridized at 60 °C in 50 mM PIPES buffer (pH 6.4), 0.1 M NaCl, 50 mM phosphate buffer, 2 mM EDTA, 5% SDS, and 100 μg/ml herring sperm DNA. Blots were hybridized initially to a 0.6-kb *Ava*I fragment, representing the 5′-coding sequence of the cloned CHO *FUT* gene and, after boiling in 0.1% SDS, to a human cardiac actin probe (*Pst*I fragment) (34). DNA probes were labeled with [α-^{32}P]dCTP to a specific activity of ~10^9 cpm/μg using the Prime-It RmT dCTP-Labeling kit (Stratagene). Buffer and unincorporated nucleotides were removed by passage through a G-50 minispin column (Worthington). Oligonucleotides were labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Membranes were finally washed in 1× SSC containing 0.2% SDS at 50 °C. The blot was exposed to x-ray film at −80 °C for at least 24 h.

For ribonuclease protection experiments, total or poly(A)$^+$ RNA was used with the RPAII Ribonuclease Protection Assay kit from Ambion (Austin, TX) with minor modifications. Riboprobes were labeled by *in vitro* transcription to 1–2 × 10^8 cpm/μg RNA with [^{32}P]CTP using T7 or SP6 RNA polymerase (Promega). Approximately 10 μg of total RNA or 1 μg of poly(A)$^+$ RNA was hybridized overnight to ~3 fmol of antisense RNA probe at 45 °C. RNase T1 was added at a concentration of 90 units/ml in 220-μl aliquots. Reactions were performed at 37 °C for 50–80 min. Digestion products were denatured and separated by electrophoresis in 5 or 8% polyacrylamide gels containing 8 M urea at 250–300 V for 4–8 h. Nonspecific protection was monitored using yeast tRNA. Protected radiolabeled fragments were visualized by autoradiography.

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 Stratalinker (Stratagene), the membranes were hybridized in Rapid-Hyb buffer at 65 °C for 3 h. DNA fragments were labeled with [α-^{32}P]dCTP using Prime-It RmT kit (Stratagene) to a specific activity of 10^9 cpm/μg DNA. After hybridization, blots were finally washed at 65 °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. Before rehybridization, blots were erased by boiling in 0.1% SDS.

Reverse Transcription (RT) and Polymerase 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 slowly cooled to room temperature before adding 200–400 units of Superscript II reverse transcriptase together with First Strand Buffer (Life Technologies, Inc.), each of dATP, dCTP, dGTP, dTTP to 1 mM and 10 mM dithiothreitol. Reactions were incubated for 2 h at 42 °C, heated for 5 min at 95 °C, and stored at −20 °C.

PCR reactions were performed using the Expand Long Template PCR System (Boehringer Mannheim) following the protocol provided by the manufacturer. For amplification of small fragments (<3 kb), PCR buffer 1 (50 mM Tris-HCl, pH 9.2, 16 mM $(NH_4)_2SO_4$, 1.75 mM $MgCl_2$) and 350 μM of each dNTP were used; for amplification of relatively long products PCR buffer 2 (50 mM Tris-HCl, pH 9.2, 16 mM $(NH_4)_2SO_4$, 2.25 mM $MgCl_2$) and 500 μM of each dNTP were used. Two separate mixes for each reaction were prepared on ice as follows: master mix 1 (up to 25 μl) contained upstream and downstream primers (15 pmol of each), template DNA, and dNTPs; master mix 2 (up to 25 μl) contained PCR buffer, and 0.75 μl of the two thermostable DNA polymerases, *Taq* and *Pwo*. For PCR the mixes were combined in a single thin wall tube, overlaid with 30 μl of mineral oil, and heated at 94 °C for 2 min before starting the PCR program. In general the denaturing step was set at 94 °C for 20 s; annealing was for 30 s, with the annealing temperature 55–65 °C, depending on the melting temperature (T_m) of the primer pair; the elongation temperature was always 68 °C, and elongation time varied according to the expected length of PCR products. After 10 cycles, the elongation time was extended for 20 s per cycle. The primer pairs are given in figure legends. To clone, freshly made PCR products were separated on low melting agarose gels and purified using Wizard PCR Preps DNA Purification System (Promega). This DNA was subcloned into the pCRII or pCR2.1 vector using the Original TA Cloning Kit from Invitrogen.

5′- and 3′-Rapid Amplification of cDNA Ends (RACE)—For 5′-RACE, first strand cDNA synthesis was performed on 2 μg of poly(A)$^+$ RNA, using the antisense primer given in the respective figure legend. After reverse transcription, 1 μl of RNase 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 first round PCR amplification, 10 μl of cDNA product was used with oligo(dT)/anchor primer GATCAGAATTCAGCGGCCGCACC$(T)_{19}$ and the relevant gene-specific nested primer at an annealing temperature of 58 °C and an elongation time of 1 min. For second round PCR amplification, 5 μl of first round PCR product was added to the anchor primer GATCAGAATTCAGCGGCCGCACC and a second nested primer, the annealing temperature was 65 °C and the elongation time was 1 min. After second round PCR, the strongest band observed by ethidium bromide staining was excised from the gel, further gel-purified, and subjected to TA cloning (Invitrogen).

For 3′-RACE, poly(A)$^+$ RNA from each LEC11 mutant, and the oligo(dT)/anchor primer GATCAGAATTCAGCGGCCGCACC$(T)_{19}$ were used. After the RT reaction, the sample was diluted to 100 μl and purified through a Sephadex G-50 column (Boehringer Mannheim). To amplify 3′ cDNA ends, PCR reactions were carried out using 5 μl of purified first strand cDNA products with a gene-specific primer (see figure legends) and GATCAGAATTCAGCGGCCGCACC. The annealing temperature was 65 °C, and the elongation time was 1 min. The most intense ethidium bromide products between 0.5 and 1.2 kb were gel-purified and subjected to TA cloning and sequencing.

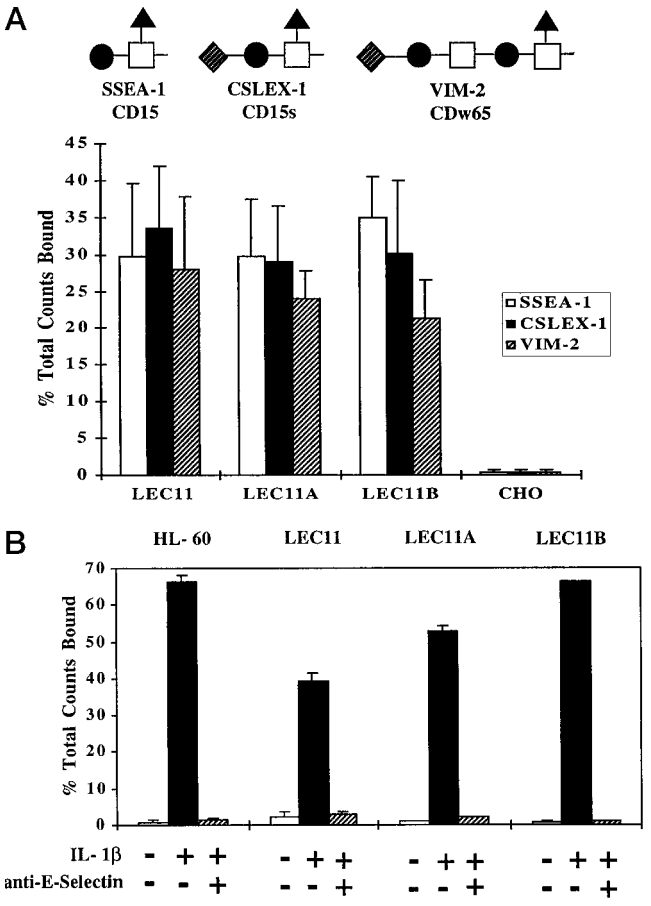

FIG. 1. **LEC11 mutants express sialyl-Le^x and recognize E-selectin.** *A,* CHO cells and the LEC11 mutants were incubated with the monoclonal antibodies shown above, and binding was determined in triplicate (± S.D.) using rabbit anti-mouse IgM and ^{125}I-protein A as described under "Experimental Procedures." Gal (●), sialic acid (◆), GlcNAc (□), fucose (▲). *B*, the LEC11 mutants and HL-60 human myeloid cells labeled with ^{51}Cr were incubated on 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."

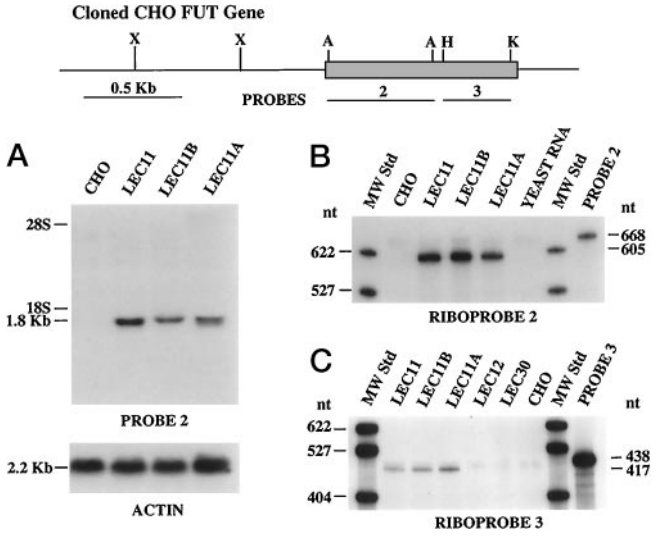

FIG. 2. **Each LEC11 mutant expresses the cloned *FUT* gene.** The *diagram* shows a partial restriction map of the cloned CHO *FUT* gene (*X*, *Xba*I; *A*, *Ava*I; *H*, *Hin*dIII; *K*, *Kpn*I) with the coding region *shaded*. Probes 2 and 3 were generated by subcloning. *A,* Northern analysis of 15 μg of total RNA for each cell line probed with probe 2 (*upper panel*) and subsequently with a probe for actin (*lower panel*). *B,* ribonuclease protection using total RNA and riboprobe 2. The protected fragment (605 nt) is only present in LEC11 mutants. *C,* ribonuclease protection with riboprobe 3 using poly(A)$^+$ RNA. The protected fragment of 417 nt was present only in LEC11 mutants. The size marker used in *B* and *C* is a *Msp*I digest of pBR322.

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 Le^X using the anti-Le^X 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 Le^X, sialyl-Le^X, and VIM-2 at similar levels to LEC11 cells (Fig. 1*A*) and, like LEC11 cells, bound E-selectin expressed on activated human umbilical vein endothelial cells (Fig. 1*B*). Fucosyltransferase assays showed that the α(1,3)Fuc-T of both new LEC11 mutants was inhibited >97% by 3 mM NEM as observed previously for LEC11 cells, and like LEC11 cells, they transferred fucose to LacNAc, sialyl-LacNAc, and 2′-fucosyl-LacNAc but not to the Type 1 acceptor, Galβ(1,3)GlcNAc, and poorly to 2′-fucosyllactose (15, 17, 24, 26; data not shown). Therefore, the LEC11 α(1,3)Fuc-T was not similar to human Fuc-T III which prefers Type 1 over Type 2 acceptors (9, 35) but was most similar to human Fuc-TV and Fuc-TVI based on acceptor specificity (36).

Isolation of a CHO FUT Gene That Is Expressed in LEC11, LEC11A, and LEC11B Cells—A λFIXII genomic DNA library prepared from Lec1 CHO cells (58) was screened with a human *FUT*3 gene coding region probe to obtain phage A6.1. An ~9-kb *Eco*RI fragment and a 3.2-kb *Sac*I subclone were co-transfected with pSV2neo into parental Pro$^-$5 CHO cells that lack endogenous α(1,3)Fuc-T activity. Both clones gave more than 30% G418-resistant transfectants which bound αSSEA-1/sRBC, 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 *Sac*I fragment contained a long open reading frame homologous to the coding region of the human *FUT*5, *FUT*3, and *FUT*6 genes (73, 72.1, and 71.9% identical respectively) but considerably different from the *FUT*4 (50.8% identical), *FUT*7 (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. 2*A*). 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. 2*B*) and the 3′-riboprobe of 415 nt protected a sequence spanning nt 638–1053 of the *FUT* gene coding region (Fig. 2*C*). No transcripts were protected in parent CHO cell poly(A)$^+$ RNA. In addition, other gain-of-function CHO mutants that possess a biochemically distinct α(1,3)Fuc-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. 3*A*). Hydropathy analysis (38) revealed a single hydrophobic membrane spanning domain of 20 amino acids near the N terminus, which

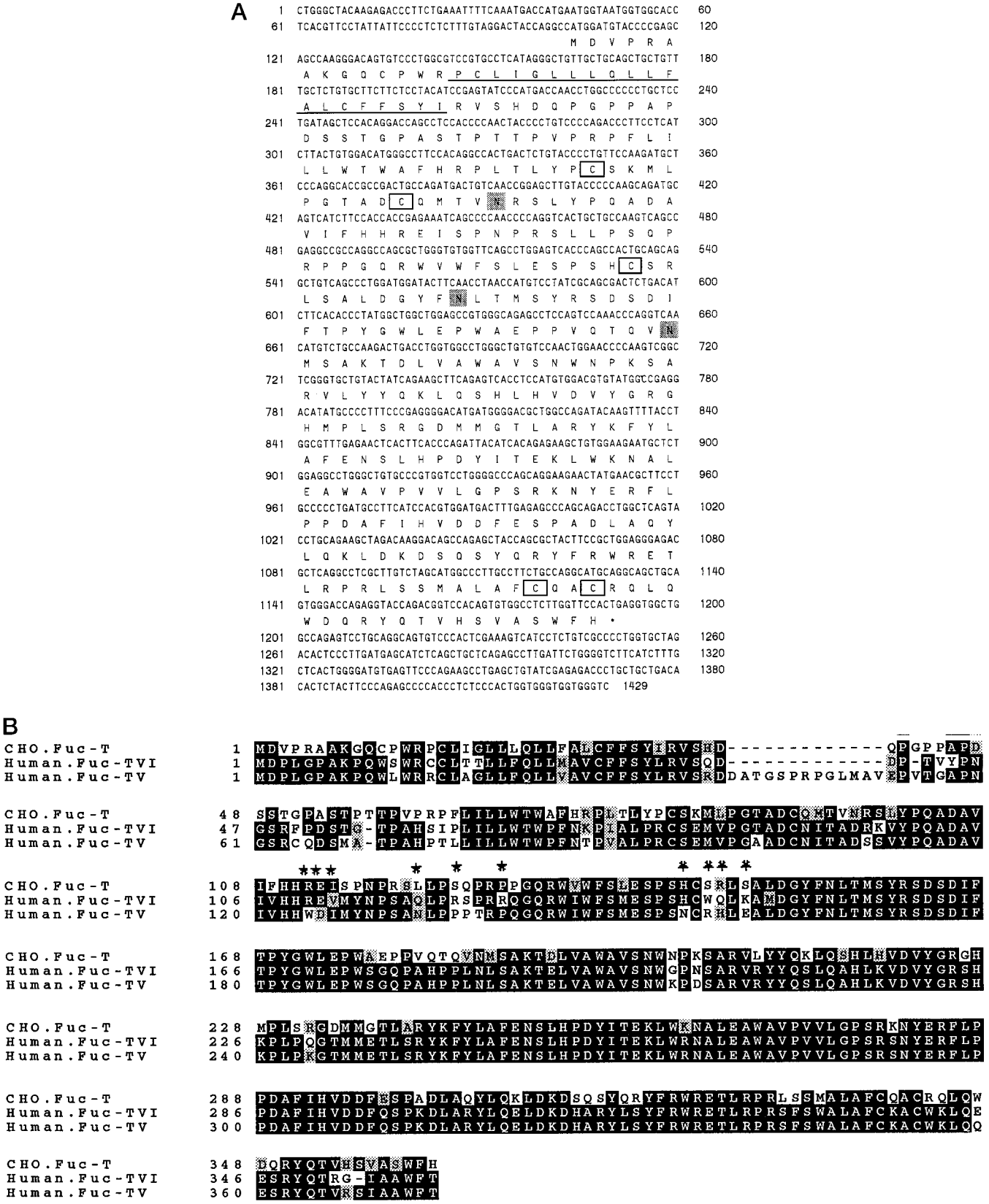

FIG. 3. **Sequence of the cloned CHO *FUT* gene.** *A,* the nucleotide sequence and predicted amino acid sequence of the CHO *FUT* gene cloned from Lec1 genomic DNA are shown. The putative transmembrane domain identified by Kyte Doolittle hydropathy analysis (38) is *underlined*. Conserved Cys residues 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-TV (accession number M81485) and 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.

TABLE I
Each LEC11 α(1,3)Fnc-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 milliunits 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 [^{14}C]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 corresponds to 100% were as follows: LEC11B, 23; LEC11A, 44; LEC11, 24; hFuc-TV, 49; hFuc-TVI, 144.

Acceptor	α(1,3)Fuc-T activity				
	hFuc-TV	hFuc-TVI	LEC11B	LEC11A	LEC11
			%		
Galβ(1,4)GlcNAc	100	100	100	100	100
SAα(2,3) Galβ(1,4)GlcNAc	302	228	112	178	153
Fucα(1,2) Galβ(1,3)GlcNAc	443	3	<1	≤2	≤5
Fucα(1,2) Galβ(1,4)Glc	188	10	<1	<1	<1

predicts the Type 2 transmembrane topology typical of mammalian glycosyltransferases (39). The sequences also predict three *N*-linked glycosylation sites, each located at a position similar to *N*-glycosylation sequons in the human *FUT*5 and *FUT*6 genes and in a bovine *FUT* gene (35). The CHO and b*FUT* genes lack a fourth *N*-glycosylation sequon at position 46 (h*FUT*5) or 60 (h*FUT*6). A comparison of the deduced CHO *FUT* gene amino acid sequences with the most related human α(1,3)Fuc-T sequences reveals that the CHO α(1,3)Fuc-T is 83.9% similar to Fuc-TVI, 81% to Fuc-TV, and 84% to Fuc-TIII. The distribution of Cys residues is conserved in human, bovine, and Chinese hamster α(1,3)Fuc-Ts, a key structural feature of NEM-sensitive fucosyltransferases (40). Cys^{144} of the CHO α(1,3)Fuc-Ts, is likely to be the Cys that is protected from NEM inactivation by GDP-fucose, analogous to Cys^{143} of human Fuc-TIII, Cys^{156} of human Fuc-TV and Cys^{142} of human Fuc-TVI.

CLUSTAL W analysis showed the CHO *FUT* gene coding sequence to be most related to the coding sequence of the h*FUT*6 gene (Fig. 3*B*). The CHO α(1,3)Fuc-T lacks the 11-amino acid insert (position 47–57) characteristic of hFuc-TV (41) and missing from the hFuc-TVI sequence (36) and the bovine α(1,3)Fuc-T (35). In addition, the CHO α(1,3)Fuc-T contains some features of a sequence identified as "unique" to the human Fuc-TVI (*starred* amino acids in Fig. 3*B*) and postulated to confer acceptor preferences (42). When the acceptor preferences of the α(1,3)Fuc-T expressed in each LEC11 mutant were compared directly with those of recombinant hFuc-TVI and hFuc-TV, it was apparent that the CHO α(1,3)Fuc-T is most similar to hFuc-TVI (Table I; Refs. 35, 43, and 44). Whereas recombinant hFuc-TV transferred fucose to fucosylated Type 1 and 2′-fucosyllactose acceptors better than to LacNAc, hFuc-TVI and the LEC11 mutants essentially did not utilize these acceptors under these conditions. Based on sequence and functional relationships therefore, the cloned CHO α(1,3)Fuc-T expressed in each LEC11 mutant represents a Chinese hamster (*C. griseus*; cg) orthologue of the h*FUT*6 gene.

Rearrangements of a CHO FUT6 Gene in LEC11 and LEC11A Cells Indicate a cis Mechanism of Gene Activation—To determine if the CHO *FUT*6 gene was rearranged in any of the LEC11 mutants, Southern analyses were performed using coding region probe 2 and the locus-specific probe 1 immediately upstream of the coding region (Fig. 4). Following *Eco*RI or *Hin*dIII digestion, locus-specific probe 1 detected a single hybridizing band in DNA from CHO cells and each LEC11 mutant (Fig. 4*A*). However, the same blot hybridized to coding region probe 2 gave two or three hybridizing bands, the largest one being the same size as the fragment detected by probe 1. Six different restriction enzymes gave at least two hybridizing fragments with coding region probe 2. Fragments of identical size were present in Chinese hamster liver DNA (Fig. 4*A* and

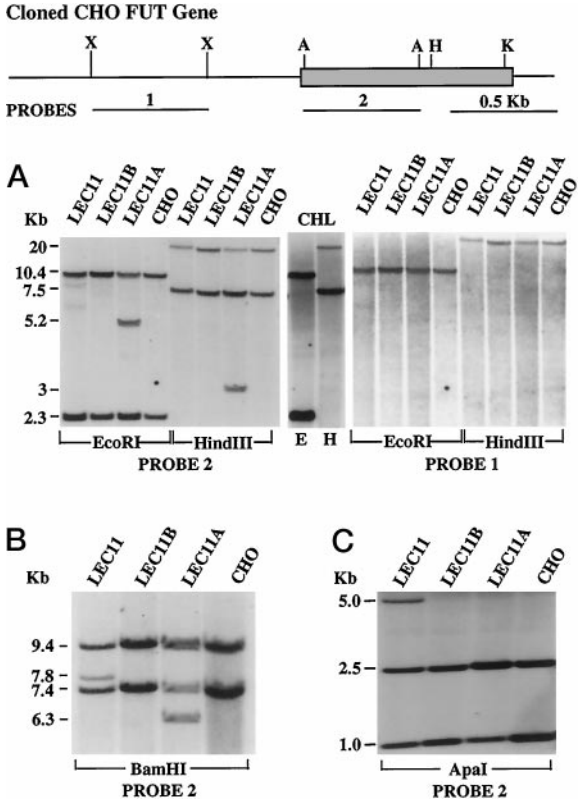

FIG. 4. **Rearrangements of a cg*FUT*6 gene in genomic DNA from LEC11 and LEC11A cells.** The diagram shows a partial restriction map of the cloned cg*FUT*6 gene (*X, Xba*I; *A, Ava*I, *H, Hin*dIII; *K, Kpn*I) with the coding region *shaded*. For Southern analysis, 15 μg of genomic DNA was digested with 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*, *Eco*RI; *H*, *Hin*dIII. *B,* DNA digested with *Bam*HI and hybridized to probe 2. *C,* DNA digested with *Apa*I 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 cg*FUT* 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 cg*FUT*6 gene. Five of six restriction enzymes examined detected an extra fragment in LEC11A DNA (*Eco*RI, 5.2 kb; *Hin*dIII, 3 kb; *Bam*HI, 6.3 kb; *Bgl*II, 9.7 kb; *Kpn*I, 6.5 kb) and three detected an extra fragment in LEC11 DNA (*Bam*HI 7.8 kb; *Kpn*I, 7.2 kb; *Apa*I, 5 kb). Therefore, one copy of a CHO cg*FUT*6 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 cg*FUT*6 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 *FUT*6 gene. However, no rearrangements were detected in genomic DNA from LEC11B cells. Therefore, the mechanism of activation of a cg*FUT*6 gene in this mutant was examined by somatic cell hybrid analysis.

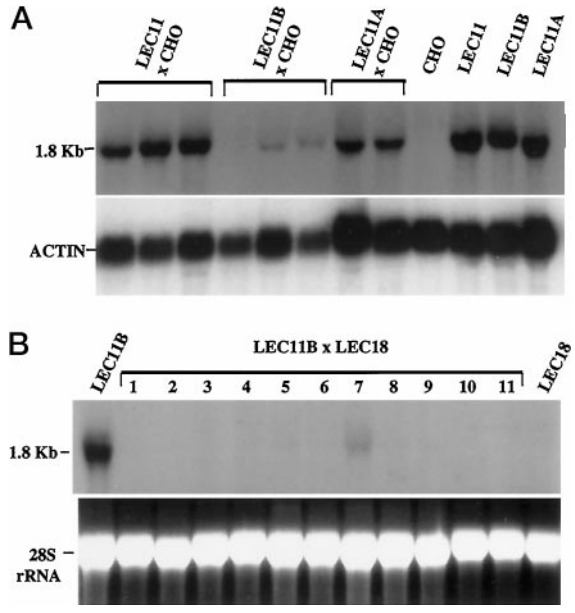

FIG. 5. **Suppression of cg*FUT*6 gene transcripts in LEC11B hybrids.** *A,* total RNA (10–15 μg) from independent CHO cell hybrid lines (derived from fusing the designated pairs of cells) or from CHO cells and the LEC11 mutants were electrophoresed, transferred to membrane, and hybridized to probe 2 (*top panel*). Blots were subsequently stripped and hybridized to an actin probe (*lower panel*). *B,* total RNA from 11 independent hybrids obtained from fusing LEC18 and LEC11B cells was electrophoresed, transferred to membrane, and hybridized to probe 2 (*upper panel*). Ethidium bromide staining showed that approximately equal amounts of RNA were loaded.

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 cg*FUT*6 gene in LEC11B cells, somatic cell hybrids were analyzed for the ratio of cg*FUT*6 gene transcripts compared with actin transcripts. In a *cis* mechanism of gene activation, a rearranged cg*FUT*6 allele would continue to be transcribed in hybrids formed with CHO cells, and the cg*FUT*6 genes in the CHO genome would remain silent, giving a cg*FUT*6 transcript:actin transcript ratio close to 0.5. In a *trans*-positive mechanism, a gene other than a cg*FUT*6 gene would be affected, and its product would activate a cg*FUT*6 gene in the mutant *and* in the parent CHO genomes. In this case, the cg*FUT*6:actin transcript ratio would be close to 1.0. In a *trans*-negative mechanism of gene activation, the negative regulator that keeps cg*FUT*6 genes silent in CHO cells would suppress the cg*FUT*6 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. 5*A* show that LEC11 × CHO and LEC11A × CHO hybrids contained readily detectable cg*FUT*6 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 cg*FUT*6 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 cg*FUT*6 gene transcripts by Northern analysis (Fig. 5*B*). The presence of low levels of cg*FUT*6 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 $\sim 10^{-4}$ per cell per generation (45).

TABLE II
Ratio of cgFUT6:actin gene transcripts in hybrids

Northern blots similar to those in Fig. 5 were subjected to densitometry, and the ratio of arbitrary units for cgFUT6:actin was determined.

Cells fused	No hybrids tested[a]	cgFUT6:actin transcripts observed[b]	Ratio predicted for *cis*[c] mechanism
LEC11 × CHO	5	0.65 ± 0.24 (n = 21)	0.5
LEC11A × CHO	4	0.29 ± 0.13 (n = 10)	0.5
			Ratio predicted for *trans*[c] mechanism
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

[a] Number of independent hybrids tested.
[b] Average and standard deviation of densitometry results of cg*FUT*6 gene transcripts:actin gene transcripts. n = number scanned.
[c] Predicted value of densitometry result of each hybrid for a *cis-* or *trans*-mechanism of cg*FUT*6 gene expression.

TABLE III
α(1,3)Fuc-T assays of LEC11B × CHO hybrids

The substrate used in the α(1,3)Fuc-T assays was Galβ(1,4)GlcNAc (LacNAc).

Cell line	Exp.	α(1,3)Fuc-T activity	
		Observed[a]	Predicted[b]
		pmol/min/mg protein	
CHO	1	0.6	
	2	<2.1	
LEC11B	1	115.3	
	2	229.3	
LEC11B × CHO	1	20.0	115.9
	2[c]	<2.2	231.4
	2	12.7	231.4
	2	67.7	231.4

[a] Observed α(1,3)Fuc-T activity from cell lysates.
[b] Expected α(1,3)Fuc-T activity for each cell hybrid, assuming activities are additive.
[c] In experiment 2, three independent hybrids were assayed.

The ratio of cg*FUT*6: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 cg*FUT*6 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 hypersensitive to ricin. The combined data provide strong evidence that the cg*FUT*6 gene is expressed in LEC11B cells due to an inactivating mutation in a gene that encodes a negative regulatory factor.

Only One of Two cgFUT6 Genes Is Expressed in LEC11B Cells—Southern analyses showed that the CHO genome con-

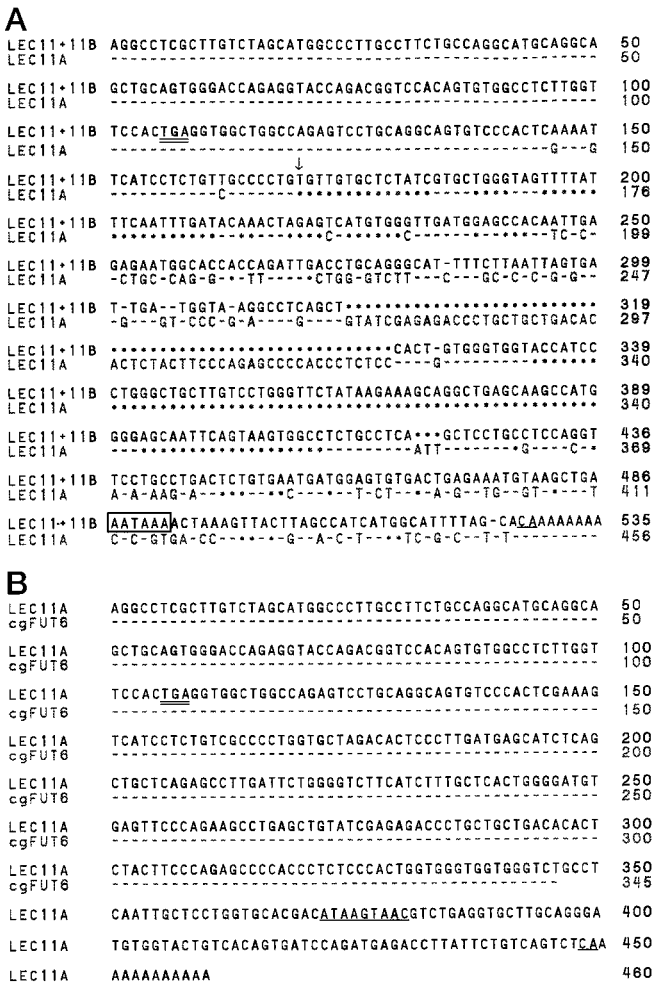

FIG. 6. **LEC11B transcripts have divergent 3′- and 5′-UTR sequences.** *A,* 3′-RACE of poly(A)$^+$ RNA from LEC11, LEC11A, and LEC11B cells using the oligo(dT)/anchor primer ("Experimental Procedures") for reverse transcription. First round cDNA products were amplified with the anchor primer ("Experimental Procedures.") and primer 152 (AGGCCTCGCTTGTCTAGCATGG; sense) from the 3′ end of the cg*FUT*6 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. The 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 cg*FUT*6 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 (TGCCTGGGAGCATCTTGGAAC; antisense) near the 5′ end of the cloned cg*FUT*6 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 (CATGTCCACAGTAAGATGAG; antisense). Two 5′-RACE sequences are aligned with the sequence upstream of the cloned cg*FUT*6 gene-coding region (Fig. 3). The ATG initiation codon is *double underlined*. *D,* the coding region of the cg*FUT6* 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 AZ2 (GAGGCCACTTACTGAATTGCTCCC). PCR of cDNA products was performed with the 5′-UTR sense primer 141 (CTGCTACCCTGCAGTAGAGCTTG) and 3′-UTR antisense primer AZ1 (ACACTGCCTGCAGGACTCTGGC). The 1.2-kb PCR product was cloned, and four independent clones gave the deduced amino acid sequence shown (cg*FUT*6B). The same sequence was obtained from genomic DNA of Gat$^-$2 parental CHO cells using primer 257B (GATCCCCCAGGCCATGGAT; sense) immediately upstream of the cg*FUT6*B coding region and primer 171 (AGCTTACATTTCTCAGTCACACTCC; antisense) from the 3′-UTR region. Genomic DNA from Pro$^-$5 and Gat$^-$2 parental CHO cells was also used to obtain the sequence of the cg*FUT6*A gene with primer 257A (GGACTACCAGGCCATGGAT; sense) immediately upstream of the coding region and primer 169 (CTGACAGAATAAGGTCTCATCTGG; antisense) from the unique 3′-UTR region. This sequence differed by 2 nucleotides (G214C and G225C numbered from the ATG) and 2 amino acids (A72P and R75S) from the FUT coding region cloned from Lec1 genomic DNA (Fig. 3) but did not differ from the cg*FUT*6B gene at these positions. The combined genomic DNA and cDNA sequences of the cg*FUT*6B gene are submitted under GenBank™ accession number AF090449 and the coding region and 3′-UTR sequence for cg*FUT*6A has GenBank™ accession number AF090450.

tains two genes homologous to the cloned cg*FUT*6 gene (Fig. 4). To determine whether one or both of these genes is expressed in LEC11B cells, 3′-RACE was performed on poly(A)$^+$ RNA from each LEC11 mutant. A complete 3′-UTR sequence was obtained in each case (Fig. 6*A*). The LEC11B and LEC11 3′-RACE products differed in only three nucleotides from the sequence of the cloned cg*FUT*6 gene up to nucleotide 38 from the coding region stop codon. Thereafter, however, they diverged completely from the cloned sequence (Fig. 3) and from the sequence obtained from LEC11A 3′-RACE products (Fig. 6*A*). By contrast, LEC11A 3′-RACE products were identical to the cloned sequence in Fig. 3 and included additional sequence until a stretch of poly(A) (Fig. 6*B*). From these analyses it could be concluded that LEC11A transcripts derived from the cloned cg*FUT*6 gene, whereas LEC11 and LEC11B transcripts both derived from a distinct *FUT6* gene. Consistent with this, it was shown that a probe obtained from the unique 3′-UTR sequence common to LEC11 and LEC11B transcripts hybridized only to transcripts from those cells and not to LEC11A transcripts (Fig. 7).

When LEC11B poly(A)$^+$ RNA was subjected to 5′-RACE using a primer near the ATG of the cloned cg*FUT*6A gene, a sequence that diverged 5′ of the cloned cg*FUT*6A gene sequence was obtained, precisely at a conserved splice acceptor site (Fig. 6*B*). A probe derived from this new sequence hybridized only to cg*FUT*6 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 cg*FUT*6 gene transcripts provides further evidence for rearrangement of the respective cg*FUT*6 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 cg*FUT*6 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 cg*FUT*6 gene that is functionally expressed only in LEC11A cells and is henceforth termed cg*FUT*6A. 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 cg*FUT*6 gene, henceforth termed cg*FUT*6B.

Additional evidence for the existence of two *FUT*6 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 cg*FUT6*A and cg*FUT6*B coding regions and paired with primers from the unique 3′-UTR region of each gene (see Fig. 6*A*). With cg*FUT6*A 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

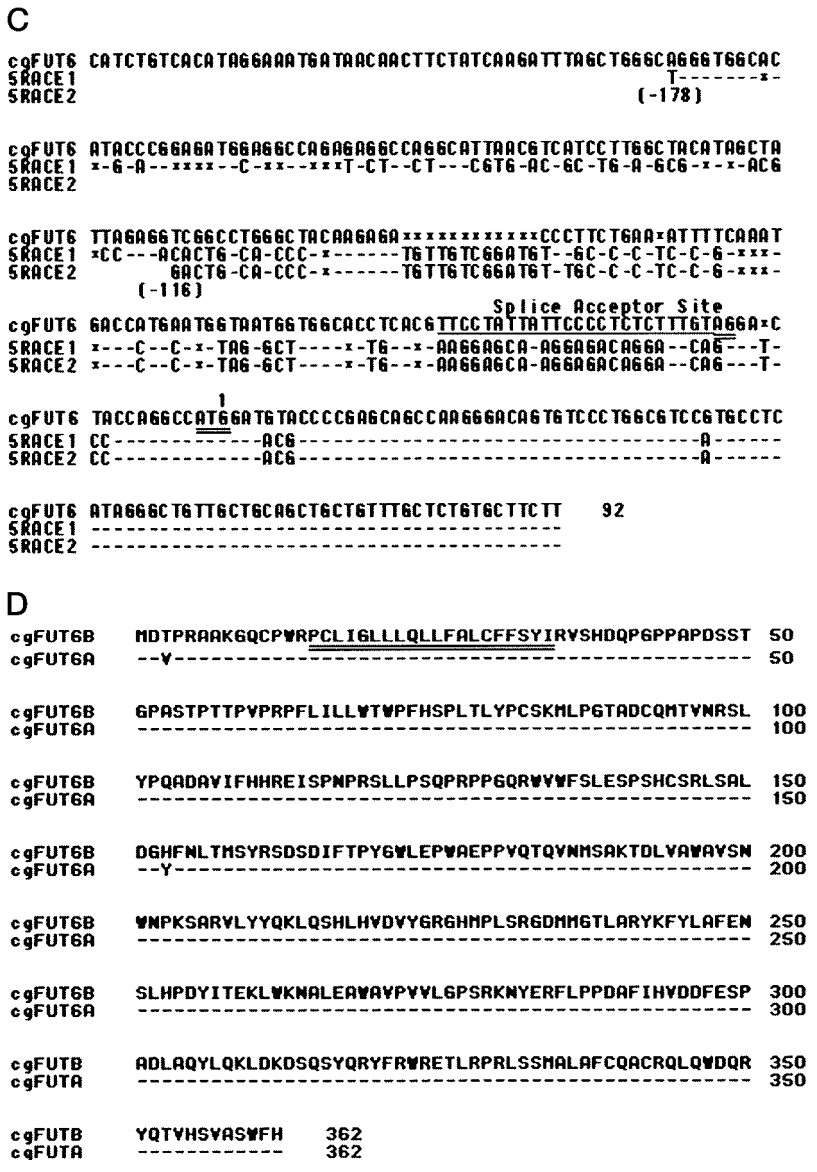

FIG. 6—*continued*

presumably reflects cg*FUT6*A gene mutations present in the Lec1 genome or that arose during cloning. These changes translate into two amino acid differences (A72P and R75S; Fig. 6*D*). With cg*FUT6*B 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 cg*FUT6*B gene differed from the cg*FUT6*A gene sequence in seven nucleotides that translated into only two amino acid differences (Fig. 6*D*).

cgFUT6B Gene Transcripts Are Suppressed in LEC11A × LEC11B Hybrids—If a negative regulatory factor represses expression of the cg*FUT*6B gene in LEC11B cells by a *trans* mechanism, and the cg*FUT*6A gene is expressed in LEC11A cells by a *cis* mechanism, hybrids formed between LEC11A and LEC11B cells should suppress expression of cg*FUT*6B gene transcripts and express mainly cg*FUT*6A gene transcripts. The Northern blots in Fig. 9 show this to be the case. Independent LEC11A × LEC11B hybrids contained predominantly cg*FUT*6A gene transcripts. Densitometry analyses of the FUT6:actin signal ratio for hybrids with the cg*FUT*6A gene-specific probe averaged 0.88 compared with 0.94 for LEC11A RNA and 0.02 for LEC11B RNA (Fig. 9*A*). By contrast, the ratio for the cg*FUT*6B probe averaged 0.1 for hybrid transcripts (Fig. 9*B*). 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 cg*FUT*6A gene which is functional in LEC11A cells, whereas transcripts from the cg*FUT*6B gene were suppressed. Consistent with this, fucosyltransferase activities of two LEC11A × LEC11B hybrids were those expected if only one cg*FUT6* gene was active. Thus, the *trans*-negative regulatory factor contributed by the LEC11A genome specifically suppressed expression of the cg*FUT*6B 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 cg*FUT*6 genes, whereas the third arose due to the loss of

an NRF that suppresses expression of only the cg*FUT*6B gene in CHO cells (Table IV). Neither cg*FUT*6 gene gave detectable transcripts in poly(A)$^+$ RNA from CHO cells. The cg*FUT*6 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 *FUT*6 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 cg*FUT*6B 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 cg*FUT*6B gene because LEC11 cells, which carry the NRF, express stable cg*FUT*6B gene transcripts (Fig. 7). It could be an NRF that binds to the 5′-UTR sequence unique to cg*FUT*6B transcripts in LEC11B cells or a splicing factor that normally splices out transcripts of the cg*FUT*6B gene. However, it seems most likely to be a negative regulator of transcription of the cg*FUT*6B 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 *FUT* 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-Glc-NAc-T gene that is regulated during mammary gland development has an NRE located ~1 kb upstream of the ATG (49, 50).

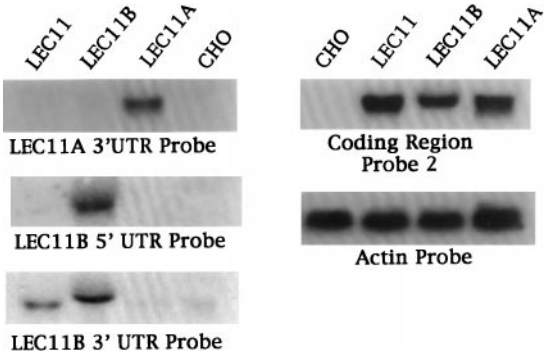

FIG. 7. **Northern analysis using gene-specific probes.** Total RNA (10–15 μg) from CHO and LEC11 mutants was electrophoresed, transferred to membrane, and hybridized to probes derived from 3′-RACE and 5′-RACE products or coding region probe 2 (Fig. 2) or the actin probe. The 5′-UTR probe specific for LEC11B transcripts was an *Eco*RI fragment of 150 bp derived from a cloned 5′-RACE product obtained by RT-PCR from LEC11B poly(A)$^+$ RNA using primer 143 (TTCTGCAGGCCAAGCTCTACTGC; antisense) based on sequence unique to the LEC11B 5′-UTR (Fig. 6*C*). 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 LEC11 and LEC11B cells was obtained by PCR of a cloned cDNA obtained by 3′-RACE from LEC11B poly(A)$^+$ RNA using sense primer 170 (TTGCCCCTGTGTTGTGCTCTATCG) and antisense primer 171 (AGCTTACATTTCTCAGTCACACTCC).

The *FUT*6 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 *FUT*6 gene silent in the tissues where it is not expressed, such as lung (11). In human pancreas the *FUT*6 gene is transcriptionally silent, but it is expressed in pancreatic tumors (53). This may reflect down-regulation of a *FUT*6 gene NRF, leading to enhanced expression. This may in turn lead to metastasis following expression of Le^X and SLe^X on cancer cells. The NRF that regulates *FUT*6B expression in CHO cells may be involved in this type of control. Although the cg*FUT*6 genes are almost identical in their coding regions, the regulation of their expression is independent because each LEC11 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 *FUT* genes silenced during development and differentiation occurs in cancer, and the expression of SLeX is associated with poor prognosis, pos-

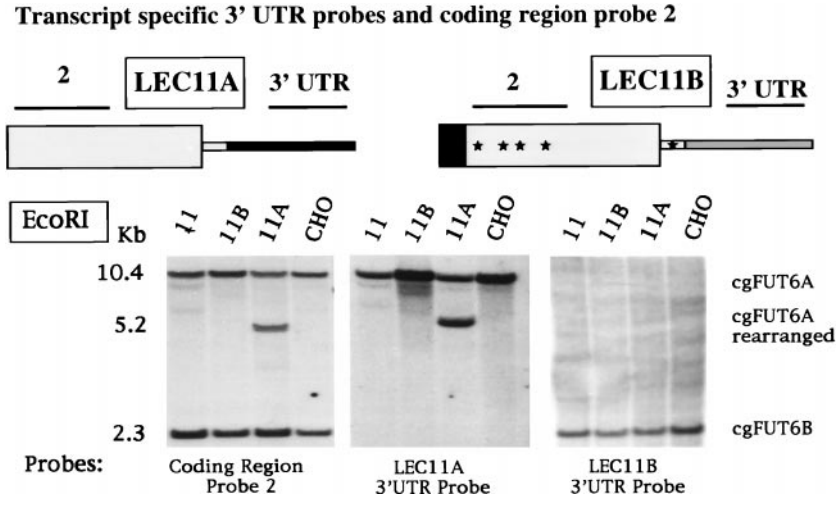

FIG. 8. **Southern analysis using gene-specific probes.** Gene-specific probes were derived from the 3′-UTR sequences unique to the LEC11A and LEC11B transcripts, respectively, as depicted in the diagram. Sequence differences between the coding regions of each transcript are denoted by *stars* in LEC11B. Genomic DNA (10–15 μg) from CHO cells and LEC11 mutants was digested with *Eco*RI, electrophoresed, transferred to membrane, and hybridized to coding region probe 2 (see diagram) or the probe specific for the 3′-UTR region of LEC11A transcripts in order to identify the cg*FUT*6A gene, or the probe specific for the 3′-UTR of LEC11B transcripts to identify the cg*FUT*6B gene (see Fig. 7). Slight degradation of LEC11B genomic DNA is apparent in the middle blot. The cg*FUT*6B gene-specific probe gave background hybridization but hybridized at high stringency only to the 2.3-kb fragment that contains cg*FUT*6B.

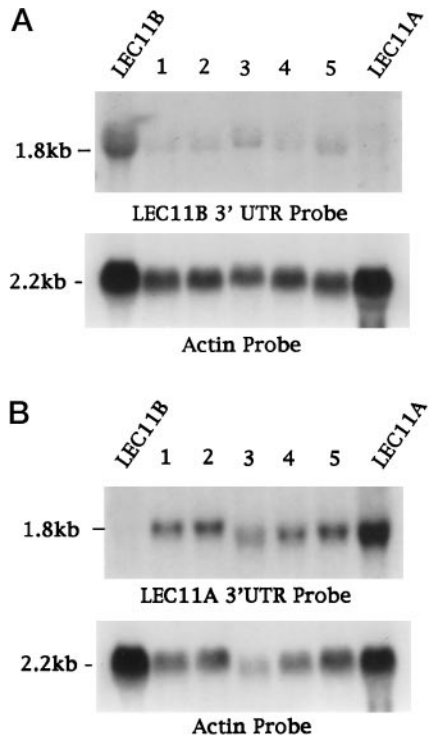

FIG. 9. **Transcripts of the cg*FUT*6B gene are suppressed in LEC11A × LEC11B hybrids.** Independent hybrids 1–5 were isolated from a fusion between LEC11A and LEC11B cells. Total RNA isolated from the hybrids or from LEC11A or LEC11B cells was electrophoresed, transferred to membrane, and hybridized with gene-specific probes (see Fig. 7) followed by an actin probe. *A,* probe specific for cg*FUT*6B transcripts expressed in LEC11B cells (*upper panel*). *B,* probe specific for cg*FUT*6A transcripts expressed in LEC11A cells. The ratio of FUT6: actin signals was determined for each lane by densitometry and is given in the text.

TABLE IV
Summary of LEC11 gain-of-function CHO mutants

GOF mutant	cg*FUT*6 gene expressed	cg*FUT*6 gene rearrangements	Activation mechanism
LEC11A	cg*FUT*6A	*Eco*RI, *Hin*dIII, *Bam*HI, *Bgl*II, *Kpn*I	*Cis*
LEC11	cg*FUT*6B	*Kpn*I, *Apa*I, *Bam*HI	*Cis*
LEC11B	cg*FUT*6B	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 *FUT*6 genes that have almost identical coding regions is of interest in terms of evolutionary relationships of related *FUT* genes. The human Lewis genes, *FUT*3, *FUT*5, and *FUT*6 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 *FUT*6 and *FUT*3 genes (54) and of ~25 kb between the *FUT*3 and *FUT*5 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 α(1,3)Fuc-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* assays (55, 56). Phylogeny 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 (*FUT*6-*FUT*3-*FUT*5) 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 h*FUT*6. 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 dendrogram[3] 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.

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